CORRELATION OF PLASMA OSTEOPONTIN WITH RADIOLOGICAL GRADING IN PATIENTS WITH OSTEOARTHRITIS IN THE KNEE JOINT

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BONAFIDE CERTIFICATE

This to certify that this dissertation work entitled **"CORRELATION OF PLASMA OSTEOPONTIN WITH RADIOLOGICAL GRADING IN PATIENTS WITH OSTEOARTHRITIS IN THE KNEE JOINT"** is the original bonafide work done by **Dr.P.Nirmaladevi**, Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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DECLARATION

I, **Dr.P.Nirmaladevi**, solemnly declare that the dissertation titled **"CORRELATION OF PLASMA OSTEOPONTIN WITH RADIOLOGICAL GRADING IN PATIENTS WITH OSTEOARTHRITIS IN THE KNEE JOINT"** is the bonafide work done by me at Institute of Biochemistry, Madras Medical College under the expert guidance and supervision of **Prof. Dr.R.Chitraa,** M.D., Professor, Institute of Biochemistry, Madras Medical College. The dissertation is submitted to the Tamil Nadu Dr. M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch XIII) in Biochemistry.

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CONTENTS

ABBREVIATIONS

1.	OA	–	Osteoarthritis
2.	MMP-13	–	Matrix Metalloproteinase-13
3.	IL	–	Interleukin
4.	TNF	–	Tumour Necrosis Factor
5.	BMI	–	Body Mass Index
6.	COMP	–	Cartilage Oligomeric Matrix Protein
7.	VDR	–	Vitamin D Receptor
8.	CD	–	Cluster of Differentiation
9.	DDR2	–	Discoidin –domain containing receptor
10.	MEK/ERK	–	Mitogen activated protein kinases/ Extracellular signal- regulated kinases
11.	ATP	–	Adenosine triphosphate
12.	PP	–	Pyrophosphate
13.	CPPD	–	Calcium pyrophosphate dehydrate
14.	TIMP	–	Tissue inhibitors of metalloproteinases
15.	TGF	–	Transforming growth factor
16.	BMP	–	Bone Morphogenetic Proteins
17.	TPA	–	Tissue plasminogen activator
18.	Hif2alpha	–	Hypoxia- inducible factor 2alpha
19.	ADAMTS	–	A disintegrin and metalloproteinase with thrombospondin motif
20.	RUNX2	–	Runt-related transcription factor 2

21.	IHH	–	Indian hedgehog signalling pathway
22.	miR	–	micro- RNA
23.	Rho/ROCK	–	Rho associated protein kinase
24.	iNOS	–	inducible isoform of nitric oxide synthase
25.	PGE2	–	Prostaglandin E2
26.	ecNOS	–	endothelial cell nitric oxide synthase
27.	KL	–	Kellgren- Lawrence grading system
28.	CTX-II	–	Carboxy- Terminal telepeptides of Type-II collagen
29.	CRP	–	C Reactive Protein
30.	OPN	–	Osteopontin
31.	SIBLING	–	**S**mall **I**ntegrin-**B**inding **L**igand **N**-linked **G**lycoprotein
32.	VDRE	–	Vitamin-D response element
33.	VSMC	–	Vascular smooth muscle cell
34.	NF-κB	–	Nuclear factor kappa-light-chain-enhancer of activated B cells
35.	HA	–	Hyaluronic acid
36.	ECM	–	Extracellular Matrix
37.	RHAMM	–	Receptor for HA mediated motility
38.	ICAM-1	–	Intercellular adhesion molecule-1

CORRELATION OF PLASMA OSTEOPONTIN WITH RADIOLOGICAL GRADING IN PATIENTS WITH OSTEOARTHRITIS IN THE KNEE JOINT

ABSTRACT:

Objectives and aims of the study:

To correlate plasma osteopontin level with radiological grade in patients with knee osteoarthritis (OA) and to correlate plasma osteopontin level with serum hyaluronic acid in patients with knee OA, thereby to assess, if osteopontin contributes to the pathogenesis of the degenerative process of OA by stimulating MMP13 and increase hyaluronic acid levels in serum.

Materials and methods:

60 patients with varying grades of radiological evidence of OA in the knee joint and 30 healthy subjects as controls were enrolled in the study. Anteroposterior knee radiographs, in standing position were taken to determine the disease severity of the affected knee joint. The radiographic grading of OA in the knee joint was performed by using the Kellgren–Lawrence grading (K/L). Osteopontin levels in the plasma and hyaluronic acid levels in the serum were measured using enzyme-linked immunosorbent assay and compared.

Results:

The mean plasma osteopontin concentration of the knee OA patients was significantly higher compared with that of healthy controls (984.91+/-804 pg/mL vs 611.05+/-207.59 pg/mL, p=0.014). The plasma osteopontin levels significantly correlated with severity of disease (r=0.349, p=0.006). The mean serum hyaluronic

acid concentration of the knee OA patients was significantly higher compared with that of healthy controls (2.58+/−0.90ng/mL vs 1.87+/−0.48 ng/mL). The serum hyaluronic acid level significantly correlated with K/L grades (r=0.358, p=0.005). The plasma osteopontin and serum hyaluronic acid levels were compared in relation to radiological K/L grades 2, 3, 4 among the cases and was found to have statistically significant higher concentrations as the grade increased with p= 0.022 for osteopontin and p=0.006 for hyaluronic acid.

Conclusion:

Osteopontin in plasma and Hyaluronic acid in serum are related to progressive joint damage in knee OA. Hence in the present study statistically significant increase in concentration of both osteopontin & hyaluronic acid with respect to radiological grade and a positive correlation between osteopontin and K/L grade & hyaluronic acid and K/L grade implies that osteopontin has a significant role in activating MMP-13 causing degradation of articular cartilage and release of HA into the circulation in OA. Hence osteopontin and hyaluronic acid can be used in combination as biomarkers to assess the severity of the disease.

Keywords:

Osteoarthritis, Osteopontin, Hyaluronic acid, Kellgren-Lawrence grading

INTRODUCTION

Osteoarthritis(OA) is a “universal disorder” affecting both sexes and all races and is the commonest of all joint diseases. OA is a “strongly age –related disorder defined by focal lesions of the articular cartilage, combined with a hypertrophic reaction in the subchondral bone and new bone formation at the joint margins with chronic nonspecific synovial inflammation”. OA is a chronic degenerative joint disease characterised by progressive destruction of articular cartilage with varying degrees of severity within a given joint.

To identify patients with a high risk for destructive OA and to monitor drug efficacy, most sensitive techniques other than “plain x-rays” are required. Hence for investigation and monitoring of patients with OA, specific and sensitive biochemical markers which reflect abnormalities in the turnover of bone, cartilage and synovial tissues may be useful.

“**Osteopontin”** is one of the major noncollageneous bone matrix proteins produced by various cells like activated T cells, macrophages, osteoblasts and chondrocytes. Osteopontin may be involved in the pathogenesis of osteoarthritis, at the molecular level[1], contributing to progressive degeneration of articular cartilage.

Osteopontin stimulates **MATRIX METALLOPROTEINASE** 13 (MMP-13), which causes bone destruction by degrading the major component of bone matrix and results in the release of hyaluronic acid into circulation.

The present study is undertaken:

1. to unravel the role of plasma osteopontin levels as a biomarker for OA and to correlate the plasma concentration of osteopontin in patients with primary knee OA with the radiological grading.
2. to understand the contribution of osteopontin in accelarating the pathogenesis of OA, anticipated to enhance serum hyaluronic acid levels.

Review of Literature

REVIEW OF LITERATURE

INTRODUCTION

OA is a "chronic degenerative disease of the joints characterised by progressive softening and disintegration of articular cartilage, hypertrophy of bone at the margins (osteophytes), cyst formation, subchondral sclerosis and a range of morphological and biochemical alterations of the capsule and synovial membrane[6] of the joint". Usually, it is distributed asymmetrically and localised to only one part of any joint and is often associated with "abnormal loading rather than frictional wear and tear".

OA is neither primarily an inflammatory disorder nor purely a degenerative disorder. OA is a dynamic phenomenon which shows features of both destruction and repair. Cartilage softening and disintegration lead to hyperactive new bone formation and remodelling. The secondary factors which influence the progress of this disorder include appearance of calcium containing crystals in the joint, ischemic changes leading to osteonecrosis in the subchondral bone in elderly people causing joint instability.

OA is a disease process that affects the entire joint that includes cartilage, synovial membrane, subchondral bone, ligaments, and peri -articular muscles. OA arises from both systemic and local factors (biochemical mediated events) producing a condition with definable morphological and clinical outcomes[2].

OA may be classified into primary of unknown etiology and secondary with identifiable cause. Common causes of secondary OA include –

1. Metabolic (calcium crystal deposition, acromegaly)
2. Traumatic (joint injury)
3. Anatomic (congenital hip dislocation)
4. Inflammatory disorder (septic arthritis, ankylosing spondylitis).

Usually, secondary OA arises due to inflammatory cause with release of degenerative enzymes from synovium attributing to mechanical attrition of biomechanically altered extracellular matrix.

ETIOLOGY

The factors affecting degree of risk of developing OA are age, gender, obesity, joint location, genetic predisposition.

1. **AGE** – the most common risk factor which strongly correlate with OA. Chondrocytes undergo age-related decrease in mitotic and synthetic activity. They show a decreased response to anabolic growth factors and synthesize less uniform large aggregating proteoglycans with fewer link proteins. Age is an independent risk factor as it predisposes articular chondrocytes to apoptosis, because in aged cartilage there is higher level of expression of pro -apoptotic genes (Fas, Fas L, caspase - 8, p 53)[3].

2. **GENDER** – Women develop OA twice more commonly than men after the age of 50 due to postmenopausal oestrogen deficiency. Nuclear oestrogen

receptors are found in articular chondrocytes of humans[8] and human growth plate chondrocytes. Wluka & colleagues[9] reported that women using long term ESTROGEN REPLACEMENT THERAPY (ERT) presented with more knee cartilage than controls.

3. **OBESITY** – Another important risk factor for OA is obesity. Increased risk of OA knee is associated with increased body mass index (BMI) in both men and women. In women due to more total body fat, they tend to develop OA more commonly than men. This is because adipose tissue is a metabolically active contributor to inflammatory cascades leading to increased synthesis of pro-inflammatory cytokines- IL-1&6, TNF, leptin , adiponectin , resistin[4,5]. Hence by reducing BMI, symptoms and radiological progression can be reduced.

4. **JOINT LOCATION** – OA most commonly occur in weight-bearing joints and joint specific age related viability in articular cartilage explains why OA is more common in knee and hip, than ankle, as age advances.

5. **GENETIC PREDISPOSITION** – Genetic contribution to the pathogenesis of OA is difficult to analyse because of the prevalence in the general population and extensive clinical heterogeneity. Increased risk of OA has been associated with multiple gene variations[6] caused by mutations in genes coding for the various types of collagen. The types of collagen expressed in cartilage includes types II, IV, V, VI and CARTILAGE OLIGOMERIC MATRIX PROTEIN (COMP)[6]. The haplotype of

VITAMIN D RECEPTOR (VDR) plays a vital role in controlling bone mineral density and appears to be associated with a twofold risk of knee OA[7]. Loughlin & colleagues provided evidence that the IL-1 gene cluster harbours susceptibility to knee OA.

Studies of differential gene expression help to elucidate the pathogenesis for arriving with newer therapies and helps in

1. Identification of unique biomarkers for diagnosis and management of OA.
2. Identification of candidate susceptibility genotypes like polymorphic variations of cytokines or growth factors that may predispose to disease progression[6].

PATHOGENESIS

CHANGES IN OSTEOARTHRITIS

Morphologic changes

In early OA articular cartilage becomes irregular and roughened, in the synovial tissues, superficial clefts become apparent. As disease progresses clefts deepen and there is an increase in the surface irregularities and articular cartilage ulcerates exposing the underlying bone. On further progression of disease, the joint articulates on exposed bone causing thickening of bone and eburnation which becomes more metabolically active in response.

Early Reparative, Proliferative & Hypertrophic changes

In healthy cartilage, chondrocytes are quiescent but proliferate in clusters in the early stages of OA, associated with, expression of high levels of matrix proteins like type II collagen, aggrecan, stem cell markers and markers of hypertrophic differentiation [10]. Chondrocyte clusters are thought to contribute to the pathogenesis of OA and its progression through release of matrix degrading enzymes, inflammatory cytokines and growth factors which further affect the surrounding chondrocytes and joint tissues[11].

Osteophyte formation

Osteophytes are newly formed fibrocartilage and bone. They are commonly formed at the peripheral margins of joints between the cartilage and periosteum interface. Osteophytes contribute to the stability of joints[12, 13]. As OA progresses the abundant osteophytes formed can limit movement and become painful.

Hypo-cellularity

In aging cartilage there is a reduction in cell number due to reduced synthesis, which is an important contributing factor to the initiation and progression of OA. Apoptosis in chondrocytes can be triggered by factors which are involved in initiation and progression of arthritis viz., mechanical damage or injury, changes in cell matrix interactions, oxidative stress due to nitric oxide or reactive oxygen species, impaired mitochondrial function, signal transduction pathways i.e., CD95/CD95 ligand. Finally caspases are

accelerated to complete apoptosis. To prevent secondary OA following injury, inhibition of apoptosis by interfering with caspase activation following injury, as a chondro protective intervention is being explored. Hence autophagy can be protective in cartilage and its reduction in OA corresponds with an increase in the release of apoptotic markers[14].

ALTERATIONS IN CARTILAGE MATRIX METABOLISM

In early OA, there is a significant increase in the water content of articular cartilage which causes the tissues to swell and cause biomechanical alterations in these tissues. This suggests that there has been weakening of collagen network. Type II collagen fibres have smaller diameter than that in normal cartilage and the normal tight weave in the mid zone is slackened and distorted[15] in OA.

In later stages of OA within the extracellular matrix, type I collagen concentration increases and the proteoglycan concentration falls to less than or equal to 50 per cent, with less aggregation and shorter glycosaminoglycan side chains[16, 17]. The ratio of chondroitin-4-sulfate to chondroitin-6-sulfate increases and that of keratan sulphate concentration decreases reflecting the synthesis by chondrocytes of a proteoglycan profile which is typical of immature cartilage. As the disease progresses the proteoglycan concentration in the cartilage diminishes progressively[18].

The first step in cartilage degradation is the decrease in density of proteoglycan which is at least partly reversible[19]. The decreased proteoglycan

density opens up the cartilage porosity, increases the permeability to collagenases/ proteases, exposing collagen fibrils. This initiates a vicious cycle of positive feedback loops which further promote cartilage degradation. For example, epitopes on collagen become accessible to the cell surface DDR2 receptor which increases MMP13 production by the activation of the Ras/Raf/MEK/ERK and p38 signal cascades[20]. The partially digested matrix has a cytokine like activity that enhances inflammatory response and promotes matrix degradation. The destruction of collagenous cartilage is thought to be irreversible.

Calcium crystals are commonly found in the cartilage of the elderly and crystal arthropathy often coexists with OA. PYROPHOSPHATE (PP) is produced from adenosine triphosphate (ATP) by the exoenzyme nucleoside pyrophosphohydrolase[21]. Synovial fluid of OA patients showed high levels of PP which directly correlate with the severity of joint damage[22]. Normal adult cartilage secretes little of PP but the young or proliferating chondrocytes are the major source of PP. This increased PP secretion in OA cartilage might indicate the increased chondrocyte metabolic activity towards the matrix repair[22]. The CALCIUM PYROPHOSPHATE DEHYDRATE (CPPD) may alter the biomechanical properties of the cartilage extracellular matrix which leads to cartilage breakdown. Hemochromatosis (hemosiderin), Wilson's disease (copper), gouty arthritis (monosodium urate crystals), the CPPD crystal deposition disease are examples of condition which may alter the cartilage extracellular matrix leading to either direct or indirect chondrocyte injury and

thereby increasing the stiffness of the tissue and precipitating the development of OA.

Metabolic changes

Early OA is characterised by increased synthesis of proteoglycans, hyaluronate, collagen, noncollagenous proteins and cell replication[18, 22]. The activation of chondrocytes is thought to be an attempt to repair the cartilage matrix which yields a matrix of inferior quality which is more susceptible to degradation[23]. Both anabolic and catabolic processes increase as cells attempt to repair and maintain tissue integrity and it is this imbalance between synthesis and degradation which is thought to be important in the pathogenesis of OA[22].

In later stages of OA, there is a decrease in cell number and in the synthesis of matrix with lowered quality along with the inability to blend the hyaluronic acid [16 , 24]. In addition there is an activation of matrix degrading enzymes with an overall decrease in concentration of enzyme inhibitors such as, TISSUE INHIBITORS of METALLOPROTEINASES (TIMP), in later stages of OA. The complex interaction between matrix synthesis and degradation explains why OA is slowly progressive, static by morphologic criteria, finally resulting in the overall degradation of cartilage matrix.

CONTRIBUTING FACTORS FOR OSTEOARTHRITIS

A. Anabolic factors and cartilage repair

i. **TRANSFORMING GROWTH FACTOR – BETA (TGF –β)** – It is essential for the formation and maintenance of cartilage. It affects

cartilage homeostasis by enhancing stem cell chondrogenesis to increase the pool of cells available for cartilage synthesis, thereby increase matrix production in existing chondrocytes.

TGF-β increases synthesis of anti - catabolic factors such as TIMPs that inhibit activation of latent proteinases in cartilage. TGF-β attenuates the cellular response to inflammatory cytokines (IL-1, TGF- β, TNF) [25]. In aging cells TGF-β can have opposing effects by activating MMP-13 and inducing terminal hypertrophic differentiation in chondrocytes[26].

ii. **BONE MORPHOGENETIC PROTEINS (BMP)** – structurally related to TGF-β, activate different set of receptors and intracellular signalling molecules. It influences all stages of embryonic chondrogenesis. Recent genetic evidence reports that, impaired BMP signalling with the utmost progress for BMP-14, affects OA susceptibility[27, 28]. There is a reduction in BMP- 7 in OA cartilage with possible regulation occurring through both inhibitory microRNA[29] and promoter methylation[30]. Supplementation of BMP-7 has reduced arthritis in experimental animals and has been proven safe in phase – I clinical trial. BMP can also enhance terminal differentiation and hypertrophy in chondrocytes, the processes that are hallmarks of OA progression.

B. Catabolic factors and cartilage degradation

Cartilage remodelling and new matrix synthesis involves a degree of proteolysis which occurs via induction of an array of proteases mainly matrix metalloproteinases (MMPs). IL-1, TNF stimulate the synthesis and secretion of proteases and MMPs in OA[19] (**Figure-1).** IL -1 is synthesized by chondrocytes as an autocrine activity and by mononuclear cells and synovial lining cells in the inflamed joint. IL -1and TNF stimulate the latent forms of the enzymes viz., collagenase, stromelysin and gelatinase, including aggrecanase and TISSUE **PLASMINOGEN ACTIVATOR (TPA)** [31]. Plasminogen either enters the matrix by diffusion from synovial fluid or synthesized by the chondrocytes. TPA converts plasminogen to plasmin, a serine proteinase which activates latent cartilage degrading enzymes. HYPOXIA- INDUCIBLE FACTOR 2 alpha (**Hif2α)** is gaining increasing attention as a downstream mediator of IL-1 and TNF induced cartilage degradation[32, 33]. In OA cartilage **Hif2 α** is a transcription factor that is strongly up regulated which directly induces the expression of many cartilage degrading enzymes like MMPs- 1, 3, 9, 12 and **A DISINTEGRIN AND METALLOPROTEINASE with THROMBOSPONDIN** motif (ADAMTS-4) and ADAMTS-5 indirectly. A high level of **Hif2α** decreases the protective role of autophagy and increases the extent of cell death and activates the RUNT- RELATED TRANSCRIPTION FACTOR 2 (RUNX2) and INDIAN HEDGE HOG pathway which further contribute to cartilage matrix degradation. The different classes of proteinases activated by these cytokines in OA are discussed below in detail.

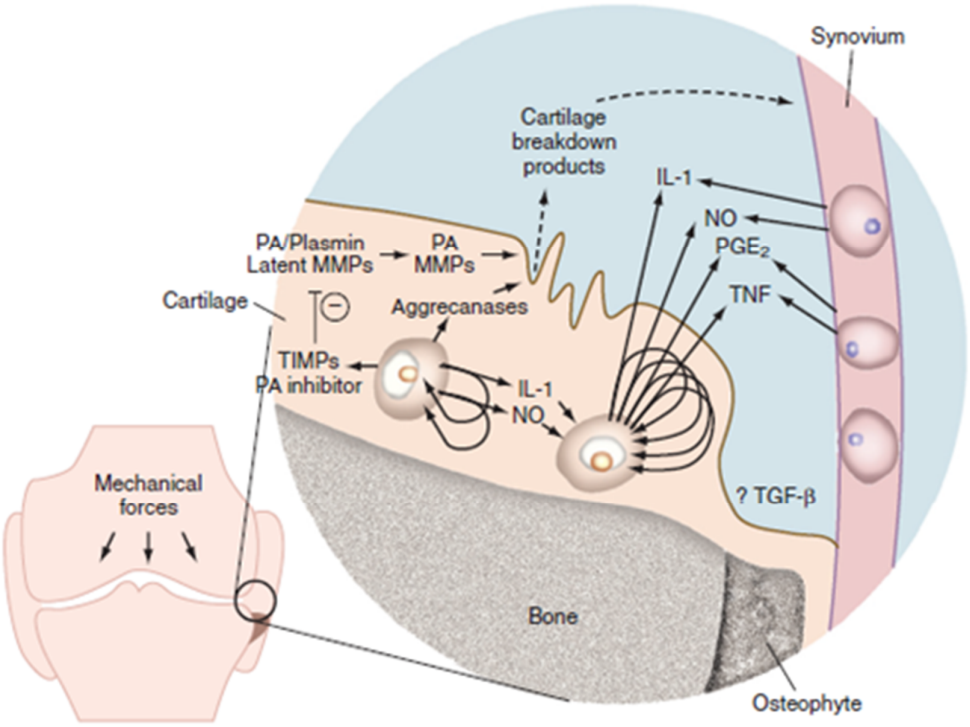


FIGURE-1-PATHOGENESIS OF OSTEOARTHRITIS

Classes of Proteinases (Metalloproteinases, Serine and Cysteine proteases, Aggrecanases)

In OA there is a marked increase in the synthesis and secretion of matrix degrading enzymes by chondrocytes[18,34]. There are four classes of proteases grouped by the catalytic mechanisms of peptide bond cleavage

1. Metalloproteinases
2. Cysteine proteinases
3. Serine proteinases
4. Aspartyl proteinases

Out of these the first three have clearly defined roles in the degradation of cartilage during the progression of OA.

Metalloproteinases

Metalloproteinases have enzymatic site which requires zinc metal ion for activity. Cartilage contains two families of metalloproteinases – ADAMTSs and MMPs. Early cartilage degeneration in OA is due to metalloproteinase enzymatic activity. Both families of metalloproteinases are up-regulated and highly expressed in OA cartilage at sites of lesion. As the MMPs and ADAMTSs play major role in the degradation of cartilage extracellular matrix and these are now acting as candidate targets for disease modification.

The control of metalloproteinase activity is complex in OA with regulation occurring at three different levels – synthesis and secretion, activation of latent enzyme and inactivation by proteinase inhibitors[35]. After

stimulation with cytokine and growth factor signalling metalloproteinases transcription is induced and its stability and translation are regulated by microRNAs. For example, microRNA -27b regulates MMP-13 expression[36]. The microRNA-140 levels are decreased in OA which reduces its repression of ADAMTS-5[37]. IL-1 and TGF-beta control these microRNAs, which are involved in the pathogenesis of OA by affecting transcript stability and protein translocation. Once translated into proteins all MMPs are expressed as inactive zymogens which require further processing for complete proteolysis. Most MMPs contain N-terminal pro-domain that blocks or inhibits the catalytic site. The primary MMP activators are serine and cysteine dependent proteases (plasminogen or pro-protein convertases and cathepsin B respectively) as well as membrane-type MMPs[38]. Activated MMPs then can be inactivated non-specifically by α2 macroglobulin and more specifically by the tissue inhibitor of metalloproteinase.

Based on their substrate specificities MMPs have been divided into three groups.

1. Collagenases – cleave all three chains of native triple helical collagen.
2. Gelatinases – cleave denatured collagen
3. Stromelysins – have broader substrate specificities[39].

There is considerable overlap in substrate between these classifications for (eg) MMP-1 (interstitial collagenases), MMP-3 (stromelysin1) and MMP-

13 (collagenase-3) all are capable of cleaving aggrecan core protein[40]. In addition to collagen, MMPs can degrade other cartilage extracellular matrix. MMPs can rapidly destroy cartilage completely if combined with plasmin which has the capability of activating many MMPs.

Collagenases

Collagenases cleave typically, the triple helical collagen allowing further degradation by other proteases. The first irreversible step in the pathogenesis of OA is the degradation of collagen which significantly reduces the mechanical properties of cartilage. The best studied MMPs capable of cleaving native collagen are MMP-13 and MMP-1 of which, MMP-13 may be the most important in OA because it preferentially degrades type II collagen[41]. Expression of MMP-13 is greatly increased in OA[42] and overall collagenase activity markedly increases in human OA cartilage cultures, suggesting that it is a major progressive factor in OA by degrading the cartilage matrix [43]. The resultant collagen fragments may be susceptible to further cleavage by other enzymes like MMP-2 (gelatinaseA), MMP-3, MMP-9 (gelatinaseB) and cathepsin B (a cysteine proteinase).

Aggrecanases

The aggrecanases belong to a family of extracellular proteases known as ADAMTS[44]. The two major aggrecanases involved in cartilage degradation are ADAMTS-4, predominantly associated with aggrecan degradation in human OA and ADAMTS-5 is more important in mouse[45-47]. The G1 region of

aggrecan is highly resistant to proteases but a glutamate-alanine bond within the extended region between G1 and G2 is susceptible to proteolysis to further degradation. ADAMTS-4 and ADAMTS-5 activity is detected in joint capsule and synovium and may be up-regulated in synovium through posttranslational processing. In addition several MMPs are also capable of cleaving aggrecan in vitro (MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, MMP-28). ADAMTS-7 and ADAMTS-12 both bind to and degrade COMP – a prominent non-collagenous protein in cartilage and the latter is up-regulated in OA cartilage[48, 49].

Several lysosomal enzymes that cleave both hyaluronic acid and chondroitin-6-sulfate have been implicated in OA progression .In the human genome there are about 6 or 7 potential hyaluronidases of which hyaluronidase-1, hyaluronidase-2, hyaluronidase-3 are likely to be active in cartilage[50]. The decrease in chondroitin sulphate chain length in OA cartilage, may be due to digestion by synovial fluid hyaluronidase which diffuse into the matrix as the permeability increases. The concentration of hyaluronic acid in OA cartilage is low in spite of the increased rate of synthesis of hyaluronic acid. These degradative enzymes disrupt the proteoglycan aggregate. The early change that occurs due to the MMP induced tissue degradation is the thinning of collagen fibres, loosening of the tight collagen network and the consequent cartilage matrix swelling in OA.

Enzyme Inhibitors (Tissue Inhibitor of Metalloproteinases, Plasminogen Activator Inhibitor – 1)

The balance between the active and the latent enzymes is controlled by two enzyme inhibitors – tissue inhibitor of metalloproteinases (TIMP) and plasminogen activator inhibitor -1 (PAI- 1)[51]. Under the regulation of TGF-β the synthesis of these two enzymes are increased[51]. If these enzyme concentrations decrease along with active enzyme concentration then the matrix degradation increases. Genes that showed increased expression in OA are MMP-2, MMP-9, MMP-13, MMP-16, MMP-28, ADAMTS-2, 12, 14, 16 and TIMP-3. Genes with decreased expression in OA are MMP-1, 3, 10 , ADAMTS-1, 5, 9, 15 and TIMP1 and 4[52]. These explain the complexity of the events that occur in the extracellular matrix regarding regulation of tissue degrading enzymes.

ALTERATIONS IN MATRIX SYNTHESIS

The changes in the extracellular matrix that occur in OA comes from the animal models. Initially there is an increase in water content in OA cartilage due to loss of elasticity of collagen network causing the hydrophilic proteoglycans to swell more than normally[51]. In early stage of OA the proteoglycan concentration may increase and the cartilage becomes thicker than normal and there is increased staining for proteoglycans[51]. At the same time water content also increases and the newly synthesized proteoglycans contain a higher proportion of chondroitin sulphate and lower proportion of keratan sulphate there by impairing the proteoglycan aggregation[51]. This

change in extracellular matrix occurs before fibrillation. As OA progresses ulcerations develop in the cartilage. Proteoglycan loss is accompanied by a decrease in ability to aggregate, persistence in abnormal glycosaminoglycan composition and also a decrease in chondroitin sulphate chain length. Once proteoglycan loss reaches a critical threshold level water content which initially increased falls below normal[53].

BIOMECHANICS AND DISEASE MECHANISMS OF OSTEOARTHRITIS

Biomechanical changes

Two biomechanical theories of pathogenesis of OA suggest that mechanical stress injure chondrocytes, release the degrading enzymes, initiate the damage of the collagen network, ultimately causing the breakdown of the matrix.

In OA cartilage the breakdown of the extracellular matrix leads to:

- loss of elasticity and compressive stiffness resulting in greater mechanical stress on chondrocytes.
- an increase in hydraulic permeability results in loss of interstitial fluid during compression and increase diffusion of solutes through the matrix.

Alterations in the inflammatory synovial fluid cause the disruption of normal fluid-film joint lubrication and loading dynamics[54, 55]. Joint friction, lubrication and contact mechanics are negatively affected by the loss of proteoglycans in the cartilage and superficial zone protein, **LUBRICIN**[56, 57].

RESPONSE OF CARTILAGE TO MECHANICAL INJURY

Mechanical injuries often result in secondary OA[58, 59]. After injury, articular cartilage produces a repair tissue with neither the original structure nor the properties of normal cartilage. Chondrocytes in areas surrounding an injured zone are unable to regenerate repair tissue with similar structure, function and biomechanical properties of normal hyaline cartilage[53]. The lack of regenerative power in the articular cartilage is the most common finding. The regenerative fibrous tissue and fibrocartilage must have originated from the undifferentiated mesenchymal tissue arising from synovium, bone marrow and the superficial layer of articular cartilage[60].

The reparative process of the avascular cartilage is not appreciated significantly, whereas the healing process in vascularized tissue, presents with three main phases viz., necrosis, inflammation and repair[58, 61]. In response to injury, cartilage undergoes initial phase of necrosis but cell death is minimal because chondrocytes are relatively insensitive to hypoxia[58, 61]. The inflammatory response is largely absent and the repair phase is limited due to vascularity. In lesions that do not cross tidemark, i.e., partial-thickness injuries, the burden of repair falls on chondrocytes – intrinsic repair[61]. Adult chondrocytes have little potential for replication and intrinsic repair. In lesions that cross the tidemark, extrinsic repair via differentiation and proliferation of mesenchymal stem cells occurs followed by a fibro cartilaginous regeneration.

The articular cartilage injury can be divided into three categories:

1. micro-damage or repetitive trauma to the cells and matrix
2. partial thickness or superficial injuries or chondral fractures that do not penetrate the sub-chondral plate
3. osteochondral (full thickness or deep penetrating) injuries that extend through the tidemark and into the underlying subchondral bone[58,61].

Trauma induces the release of proinflammatory factors (IL-1, TNF, Nitric oxide) and degrading enzymes which alter the material properties of the cartilage – cartilage matrix thins and subchondral bone stiffens accelerating the degenerative process[53].

In lesions that do not cross the tidemark within 48-72 hours, the surviving chondrocytes increase the synthesis of extracellular matrix and type II collagen accompanied by cell proliferation and formation of clusters but this is transient and it falls back to normal level resulting in suboptimal repair[53,61]. Chondrocytes proliferating on the border of injured zone do not migrate into the defect and remain unfilled by the newly synthesized matrix[53].

Lesions that cross the tidemark disrupt the underlying subchondral plate and elicit the three phase repair response like the vascularized tissues. Hence inflammatory process occurs and that stimulates a repair response. This helps in the process of fibro cartilaginous repair. The mesenchymal stem cells origin has been determined to be the underlying bone and they progressively differentiate into chondroblasts, chondrocytes, osteoblasts and synthesize

cartilage and bone matrices. After 6-8 weeks of injury, the repair tissue contains high proportion of chondrocyte like cells consisting of proteoglycans and type II collagen with less amount of type-I collagen whereas cells in the deeper layers of the defect differentiate into osteoblasts and undergo enchondral ossification to heal the subchondral bone defect. Finally there is a shift in the synthesis of collagen from type II to type I as the regenerative tissue undergoes a transformation to a more fibro cartilaginous repair. After one year of injury the repair tissue consists of a mixture of fibro cartilage and hyaline cartilage and 20-40% of type –I collagen[62]. Fibro cartilaginous repair is susceptible to early degenerative changes as it lacks the biomechanical properties to withstand normal physiologic joint loads[62].

MECHANISM OF TRANSDUCTION AND GENE EXPRESSION

Chondrocytes respond to mechanical stimuli via several regulatory pathways (e.g., transcription, translation, posttranslational modification, vesicular transport). In load-induced injury the superficial zone is more vulnerable than the middle and deep zones[63]. Normal stimuli help chondrocytes to maintain the extracellular matrix and abnormal stimuli disrupts this balance.

Mechanism of transduction influences the molecular structure of newly synthesized matrix molecules and the biomechanical tissue properties[64]. In chondrocytes cell matrix interactions via integrins are believed to be one of the important mediators in mechanotransduction. $\alpha 5\beta 1$ integrin acts as mechanoreceptor in human articular chondrocytes. Mechanical stimulation

initiates a signal cascade which involves stretch-activated ion channels, actin cytoskeleton and focal adhesion complex molecules. This result in an anabolic response manifested by increased aggrecan and decreased MMP-3 expression. Mechanical stimulation activates Rho and Rho kinase pathways that are linked to the changes in the actin cytoskeleton[65, 66]. Stimulation of Rho/ROCK pathway is an anabolic stimulus which leads to nuclear translocation and activation of Sox9 – a "master regulator" of cartilage gene expression. Indian hedgehog (IHH) protein is a key signalling molecule which controls chondrocyte proliferation and differentiation. It also acts as an essential mediator of mechanism of transduction in cartilage: IHH protein expression by chondrocytes was shown to be induced by cyclic mechanical stress.

Integrins and integrin associated signalling pathways are partly regulated by mechanical stimulation which involves activation of plasma membrane apamin-sensitive calcium activated potassium channels resulting in membrane hyperpolarization after cyclic mechanical stimulation. Following mechanical stimulation in normal cartilage chondrocytes exhibit membrane hyperpolarization to cyclic pressure induced strain where as in OA cartilage chondrocytes exhibit membrane depolarization and no changes in aggrecan or MMP-3 messenger RNA[67]. The different signalling pathways responding to mechanical stimulation in healthy chondrocytes in comparison with OA, affects the disease outcome.

Fluid flow is sensed by chondrocytes in addition to cell and matrix deformation. A study using a tissue shear loading model suggested that

deformation of chondrocytes as well as the cells around the matrix, stimulated protein and proteoglycan synthesis[68].

ABNORMALITIES OF BONE

Osteophyte Formation

Osteophytes are bony proliferations at the joint margins and in the floor of cartilage lesions that are responsible for the pain and restriction of joint movement in OA. Human osteophytes synthesize cartilage with significant amounts of type I collagen and nonaggregating proteoglycans[69]. Osteophytes are formed by penetration of blood vessels into the basal layers of degenerating cartilage or due to abnormal healing of stress fractures in subchondral trabeculae near the joint margins[69]. Subchondral cysts that occur in OA maybe created by entry of synovial fluid under pressure through defects in the cartilage or may occur in necrotic areas of the subchondral bone[69]. The increased venous pressure caused by the remodelled trabeculae and the cysts may contribute for some of the pain in OA. Glucocorticoids and immobilization have been shown to decrease the size and prevalence of osteophytes in OA of experimental models[69].

SUBCHONDRAL BONE SCLEROSIS

Early in OA increased remodelling and hardening of subchondral bone becomes evident even before the loss of cartilage thickness is evident radiologically[70]. The increased calcification results in the thinning of cartilage layer which increases the mechanical stress in adjacent areas of the cartilage. In

combination with subchondral bone sclerosis the altered mechanical environment and rapid bone remodelling may be contributing factors in cartilage degradation and OA pathogenesis.

BONE MARROW LESIONS

Studies suggest that bone marrow lesions are associated with OA. They also contribute to the pain felt by OA patients. The study authors suggest that it "remains unclear whether bone marrow lesions precede, accompany or follow cartilage damage and volume loss in OA"[71]. Unlike cartilage, bone- marrow do not show a permanent structural change in OA. Several reports suggest that these lesions either resolve or regress but it is commonly observed that the bone marrow lesion scores increases over time[72]. It has been postulated that oedema-like lesions are less severe and reversible whereas more advanced fibrotic and necrotic lesions are not[73]. There is a possibility that synovial fluid entering the subchondral bone marrow through intra-articular defects may alter the growth factor and cytokine environment that affects bone turnover. These findings suggest that there may be an altered biomechanical property of the subchondral tissues, which in turn would affect the biomechanical stress experienced by the adjacent cartilage.

ROLE OF INFLAMMATORY MEDIATORS IN DISEASE PROGRESSION

IL-1, IL-6, TNF and other classic inflammatory cytokines are elevated in the serum of patients with knee OA[74]. These cytokines auto-catalytically stimulate their own production, and thereby induce chondrocytes to produce chemokines, proteases, eicosanoids (prostaglandins and leukotrienes) and nitric oxide. Within cartilage the action of these inflammatory mediators is predominantly to activate catabolic pathways, promote cellular apoptosis and inhibit matrix synthesis. Though OA is not considered as an inflammatory response, "inflammatory" mediators from the affected tissues perpetuate disease progression and hence may represent potential targets for disease modification. Inflammatory molecules that are produced by articular cartilage include cytokines, chemokines, proteinases, nitric oxide, TGF-β, hyaluronic acid, prostaglandins, F-spondin.

NITRIC OXIDE

The chondrocytes in response to pro-inflammatory cytokines produce a major catabolic factor – Nitric oxide, produced by inducible isoform of nitric oxide synthase (iNOS) . Evidence suggests that the over production of nitric oxide by chondrocytes plays an important role in the perpetuation of cartilage destruction in OA. Without stimulation by cytokines the normal cartilage usually do not produce nitric oxide or express iNOS, but in OA cartilage they spontaneously produce large amounts of nitric oxide. iNOS is also upregulated from chondrocytes by cartilage compression[75,76].

The multiple effects exerted by nitric oxide on the chondrocytes to promote the articular cartilage degradation include the following.

1. Inhibition of synthesis of collagen and proteoglycan
2. Activation of the metalloproteinases
3. Increase in susceptibility to injury by other oxidants
4. Apoptosis

F-SPONDIN

F-spondin is a neuronal extracellular matrix glycoprotein that appears to regulate cartilage degradation through the TGF-β and PGE2 pathways. One of the recent articles suggests that addition of F-spondin in vitro to OA cartilage tissue led to increased levels of production of PGE2 as well as accelerated collagen degradation and reduced proteoglycan synthesis both of which are dependent on these two molecules[77].

ALTERATIONS IN BONE

Nitric oxide seems to have a role in OA as it contributes to the alteration in the subchondral bone. The endothelial cell nitric oxide synthase (ecNOS), endothelial isoform expressed in bone, seems to play a key role in regulating osteoblast activity and bone formation. Along with prostaglandins, ecNOS promote bone formation and suppress bone resorption. In bone cells IL-1 and TNF induce iNOS to produce nitric oxide which then potentiates the bone loss. Anabolic growth factors like IGF-1 and TGF-β are highly expressed in osteophytes in OA patients. Areas of “hot spots” increased radionuclide uptake

on bone scintigraphy identify OA joints that are more likely to progress by radiographic criteria and/or to require surgical intervention over a 5 year period.

ALTERATIONS IN SYNOVIAL TISSUE

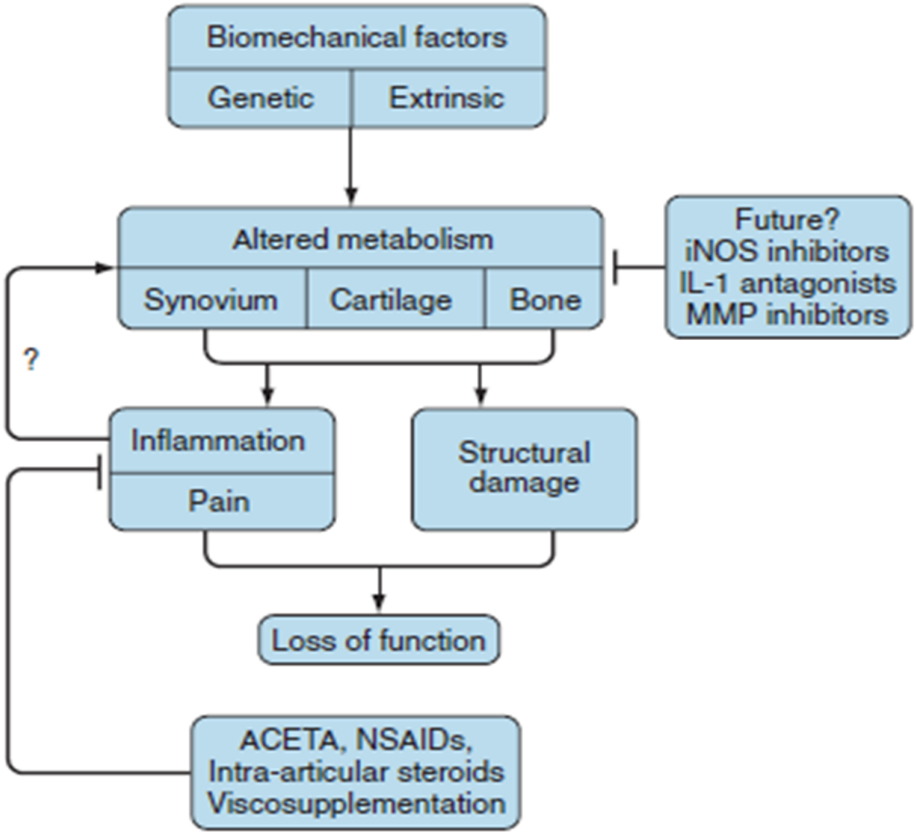
Synovial effusion and synovial lining inflammation have emerged as another key feature of OA pathophysiology. The localized inflammation of the synovium suggests that localized proliferation and inflammatory changes in the synovium occur in up to 50% of patients with OA. The activated synovium produce proteases and cytokines accelerating the damage to adjacent cartilage.

The clinical symptoms and signs in OA reflect synovial inflammation in the joints. The histologic changes in the synovium include hypertrophy and hyperplasia with an increase in the number of lining cells along with scattered foci of lymphocytes in the sub lining tissue. The synovial inflammation in OA is confined to areas adjacent to pathologically damaged cartilage and bone. These activated synovium release proteinases and cytokines, accelerating the destruction of adjacent cartilage.

Metalloproteinases that degrade the cartilage are produced not only by the cartilage but also by the synovium. The mechanical or enzymatic destruction of cartilage enhance the release of cartilage breakdown products from the articular surface and provoke the release of collagenase and other hydrolytic enzymes from synovial cells and macrophages. The cartilage

breakdown products is thought to result in vascular hyperplasia and mononuclear cell infiltration in the synovial membrane in OA.

Recent studies of arthroscopic specimens of patients with early OA revealed that synovial tissues in early OA had higher levels of IL-1β ,TNF and increased mononuclear cell infiltration when compared with late OA[78] , that are likely to be the contributors to the degradation cascade. Reports suggest that there is an increase in the number of immune cells in the synovial tissue, including B cells and activated T lymphocytes. Evidence suggests that patients with OA express cellular immunity to the C1 domain and cartilage proteoglycan link protein**. Figure-2**



Multiple factors that predispose to, initiate, and perpetuate osteoarthritis. In the future, structure-modifying treatments will be targeted to the biochemical processes that promote disease progression. ACETA, acetaminophen; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinase; NSAIDs, nonsteroidal anti-inflammatory drugs.

FIGURE-2- FACTORS PREDISPOSING TO OSTEOARTHRITIS

CLINICAL FEATURES

The joints commonly affected by OA include knee, hand, hip and spine; may be symptomatic or asymptomatic with only radiological change. Patients with OA describe pain in the joint that is worse with activity, with limited morning stiffness (<30min), pain and stiffness with rest. In OA in affected joints there is often bony enlargement and crepitus on examination and concomitant reduction in range of motion. Complaints of pain may be more or less than the expected on the basis of structural damage[79, 80].

SYMPTOMS AND SIGNS IN KNEE OA:

SYMPTOMS:

1. **Pain** – Knee OA is characterized by the insidious onset of pain with limited range of motion. They frequently describe pain and limitation with walking, transferring as from seated to standing, especially stair climbing. They are associated with a sensation of instability or "giving out" at the knee.
2. **Stiffness** – Due to stiffness, loose bodies in the joint space or meniscal lesions, there is a "locking" sensation at the knee joint.
3. **Effusions** – Effusions are usually non inflammatory that is without redness. Sometimes when large they are associated with popliteal bursa enlargement (Baker's cyst). In knee OA pain over the anserine bursa or greater trochanter may be related to altered biomechanics[81].
4. **Deformity and loss of function** – The most common mal-alignment that is often seen in severe disease is the varus deformity which may act

as a risk factor for progression[82]. Deformity may result from capsular contracture or joint instability. Difficulty in climbing stairs and restriction of walking distance eventually makes the patient to seek medical help.

SIGNS

1. **Joint swelling** – May be the first sign due to an effusion. Scars suggest previous abnormalities and muscle wasting denotes longstanding dysfunction.
2. **Deformity** - The most common deformity in knee – varus deformity.
3. **Local Tenderness** – Pain may be elicited by palpation of the medial or lateral joint line or both.
4. **Crepitus –** Patients with knee OA often have crepitus during passive movements and bony enlargement.
5. **Other signs** – include flexion deformities or joint instability.

In women an early modifiable risk factor for knee progression is quadriceps weakness[83,84] and in late stages there may be apparent muscle atrophy[85,86]. Alterations in proprioception and vibratory sense have been found to be associated with knee OA, although the relation of these factors to pain and progression is still unclear[87,88].

The most important cause that is often overlooked is patella-femoral OA that can strongly contribute to pain and disability at the knee. Patella-femoral joint OA is often located anteriorly and is characterized by pain with ascending or descending stairs. It can also be associated with tibio-femoral OA.

DIAGNOSTIC TESTING

OA is mostly diagnosed clinically, rarely laboratory testing is used. The purpose of additional diagnostic testing is to exclude the potentially treatable underlying conditions such as metabolic or inflammatory arthropathies. Before initiation of pharmacologic therapy in OA complete blood count, glucose, creatinine, and liver function tests should be obtained. Evaluation for hypothyroidism and hemochromatosis may be required in cases where there is prominent involvement of the metacarpophalangeal joints.

SYNOVIAL FLUID

The synovial fluid in OA is normal or mildly inflammatory, appearing clear/ colourless to slightly yellowish. The leukocyte cell count is less than or equal to 2000 cells/cu.mm that is less than 2cells across 10 high-power fields[89]. In case of effusion diagnostic aspiration may be done.

IMAGING: Conventional Radiography

General Considerations

Conventional radiography is a relatively inexpensive easily available technique useful to confirm the diagnosis and exclude other causes, when there is clinical uncertainty regarding the diagnosis. Joint space narrowing, sclerosis, osteophytes and cysts of subchondral bone[90] are seen in radiographs of joints affected by OA.

Imaging of the knees should be bilateral and in weight bearing (standing position) and should generally be antero-posterior (AP) for clinical purposes. The Kellgren-Lawrence (K/L) grading system remains the most commonly used one for research purposes[91]. KL grades range from 0 to 4 and a grade of 2 is generally considered diagnostic of OA.

KELLGREN AND LAWRENCE CLASSIFICATION

Grade 1: Doubtful narrowing of joint space and possible osteophytic lipping

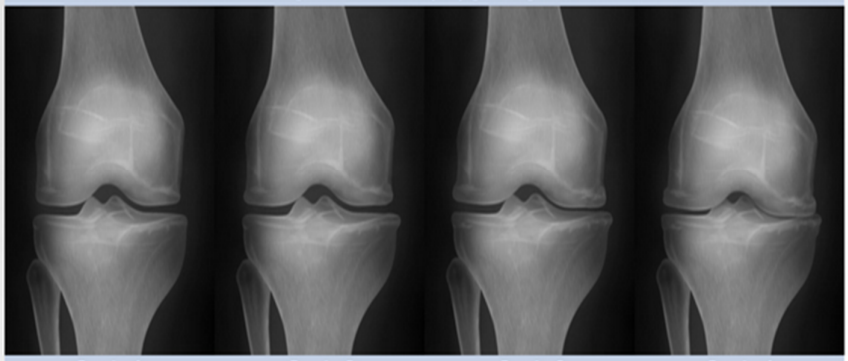
Grade 2: Definite osteophytes and possible narrowing of joint space

Grade 3: Definite narrowing of joint space, moderate multiple osteophytes, some sclerosis and possible deformity of bone contour

Grade 4: Marked narrowing of joint space, large osteophytes, severe sclerosis and definite deformity of bone contour - joint displacement.

Imaging – Radiography (X-ray)

Kellgren-Lawrence (KL) grading scale



Grade 1 Grade 2 Grade 3 Grade 4

CLASSIFICATION	Normal	Doubtful	Mild	Moderate	Severe
DESCRIPTION	No features of OA	Minute osteophyte: doubtful significance	Definite osteophyte: normal joint space	Moderate joint space reduction	Joint space greatly reduced: subchondral sclerosis

The grading system focuses on osteophyte formation, joint-space narrowing, and bone sclerosis.

Kellgren-Lawrence Grading System

Grade IV KL OA

IMAGING: Advanced Modalities

MRI is now being increasingly used in OA research as a means to obtain information about structural changes earlier in the disease process before findings are apparent on conventional radiographs. Bone marrow lesions identified on knee MRI have been shown to correlate with pain, meniscal lesions, bone attrition and progressive cartilage damage[92]. Ultrasound may have a role at the bedside in detecting small effusions, identifying early cartilage changes, differentiating inflammatory from non-inflammatory arthropathies and as a therapeutic adjunct to allow more accurate aspirations and placement of intra-articular injections[93].

BIOMARKER OF OSTEOARTHRITIS

The targets fixed in a biomarker research in OA include the following.

1. Early detection before irreversible damage occurs
2. Predicting OA progression
3. Monitoring response to therapeutic intervention

The OA Biomarkers Network funded by the National Institute of Health established the "**BIPED**" biomarker classification with five separate categories of surrogate markers: **b**urden of disease, **i**nvestigative, **p**rognostic, **e**fficacy of intervention and **d**iagnostic[94].

The burden of disease and prognosis for hip and knee OA biomarkers are serum COMP, serum hyaluronic acid and urinary CTXII[94].

The major structural components that are unique to cartilage are type II collagen and aggrecan. The proteins COMP, cartilage link protein, matrilin, minor collagens (types I, V, VI, IX, & XI), cartilage intermediate layer protein (CILP) and hyaluronic acid are other additional constituents of articular cartilage. In normal healthy cartilage there is relatively a slow turnover rate of these molecules but in OA it is characterized by enhanced synthesis and enzymatic degradation of most of these molecules. Therefore recently much focus has been on detecting biomarkers of cartilage matrix synthesis and degradation in OA[94]. In addition to markers of turnover and matrix degradation, recent advances in the proteomics and microRNA have enabled detection of new OA biomarkers using screening of serum constituents in arthritis patients[95]. The slow progression of primary OA is causing the researchers to refocus the search for OA biomarkers on secondary OA which is more rapidly progressing after acute injury[96].

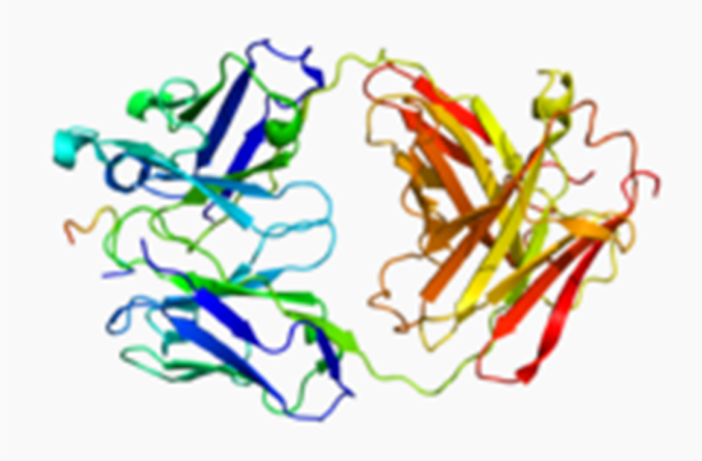
The researchers suggested that a single measurement of serum hyaluronic acid or short term increases in urine CTXII would identify patients at greatest risk for progression of OA[97]. Inflammatory markers such as CRP are moderately but significantly increased in early knee OA and can be predictive of OA that will progress over time[94].

COMP, a non-collagenous extracellular matrix protein is synthesized both by cartilage and synovium along with TGF-β, stimulation being in articular cartilage. Studies show that in knee OA serum levels of COMP are

often higher with more rapidly progressive joint damage[98]. COMP is one of the useful serum marker of OA, lacking specificity and have high variations in serum which necessitates the use of additional markers, for correlation. The development of specific reagents to detect degradation products of COMP may increase its utility as an OA biomarker[99]. Serum COMP levels indicate a specific stage of OA.

OSTEOPONTIN

Structure of osteopontin



OSTEOPONTIN (OPN) – a recently discovered ubiquitous glycoprotein, secreted by various cells of the body has been found to mineralize the bone matrix by anchoring to bone cells. OPN plays an important role in migration of neutrophil and degranulation of the mast cells. OPN is one of the newly emerging biomarker with diverse physiological role in our body. OPN is found both in body fluids as well as extracellular matrix. OPN is an acidic, phosphorylated sialic acid rich calcium binding glycoprotein with 314 amino acids. OPN was first identified by Oldberg et al[100] in 1986 in the

osteoblast cell. The human gene mapped for the osteopontin is on chromosome4q. The receptor protein contains an Arg-Gly-Asp sequence which acts as a cell adhesion sequence and recognises various integrins[101]. OPN is synthesized in fibroblasts[102], pro-osteoblasts, osteoblasts or osteoclastic cell in bone and found in kidney, specialized epithelial tissues like uterine epithelial cell, smooth muscle cell[103], skeletal muscle myoblasts[104], dentritic cells and macrophages[105]. OPN, a member of the "**s**mall **i**ntegrin-**b**inding **l**igand **N**-linked **g**lycoprotein (SIBLING) family" is known as "early T cell activation-1 protein (Eta-1)".

Experimental evidence suggests that hypocalcemia and hypophosphatemia lead to an increase in OPN transcription, translation and secretion[106] by stimulating the proximal tubule of the kidney to produce calcitriol, due to the presence of vitamin-D response element (VDRE), in the OPN gene promoter. It has been identified that extracellular inorganic phosphate is one other modulator of OPN expression[107].

Bone remodelling

The important role of OPN in bone is anchoring the osteoclasts to the bone mineral matrix[108]. The organic constituents like type I collagen, osteonectin, osteocalcin, alkaline phosphatase and OPN constitute about 20% of the dry weight of the bone.

OPN is considered essential for the initiation of the process by which osteoclasts can develop ruffled borders for bone resorption[9]. It is a product of

cells present in the osteoid matrix and forms a bridge (Latin-Pons) between cells and the mineral in the matrix[100] and hence was named as "**OSTEOPONTIN**".

Renal stone formation

In humans the primary composition of urinary stones are calcium salts. Most individuals do not form stones even though normal urine is supersaturated with calcium oxalate. This suggests that there are some factors that inhibit urinary stone formation. UROPONTIN, the urinary form of OPN has been shown to reduce growth[109] and aggregation[110] of calcium oxalate crystals .They block the binding of the crystals to the renal epithelial cells[111].

Calcium oxalate monohydrate (COM) growth and aggregation is inhibited by UROPONTIN. OPN in addition favours the formation of calcium oxalate dehydrate (COD) which is less adherent to the renal epithelial cells than COM[112].

Immune function

Thrombin cleavage modifies the full length OPN (OPN-FL) and this exposes the non-cryptic sequence SVVYGLR on the cleaved form of protein known as OPN-R. The cleaved OPN exposes epitope for the integrin receptors of $\alpha4\beta$, $\alpha9\beta4$ and $\alpha9\beta1$[113]. These integrin receptors are present in immune cells such as mast cells[108], neutrophils[114] and T cells. These are also expressed by macrophages and monocytes[115].

OPN is reported to act as an immune modulator in various ways:

1. The chemotactic property of OPN helps in promoting cell recruitment to inflammatory sites
2. Acts as an adhesion protein and help in cell attachment and wound healing
3. OPN mediates cell activation and cytokine production
4. Regulation of apoptosis by promoting cell survival

OPN is important for neutrophil migration in vitro and plays a role in migration/degranulation of mast cell[116], profoundly a macrophage chemotactic factor[117]. **(Figure-3)**

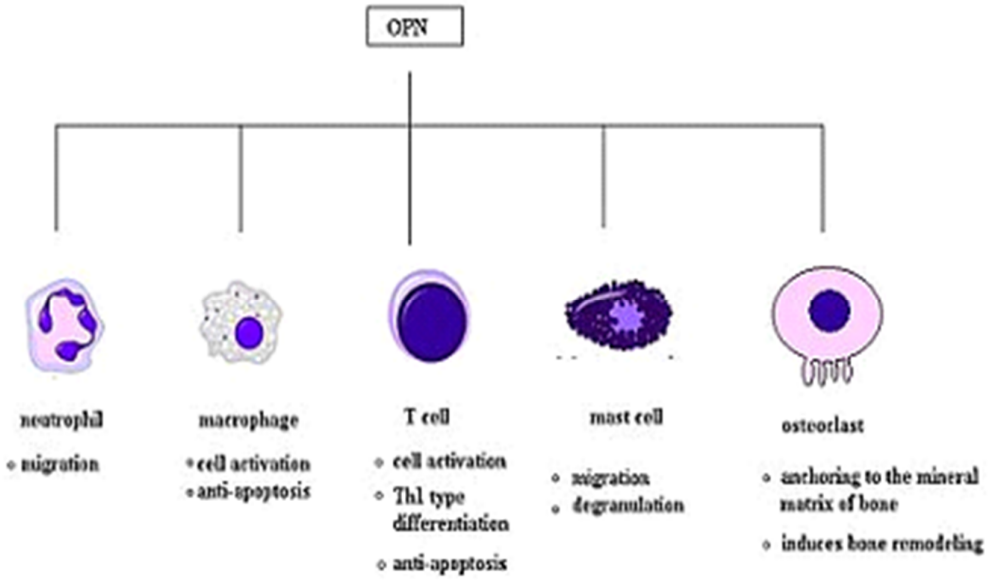


FIGURE-3- IMMUNOLOGICAL FUNCTIONS OF OPN

Cell activation

OPN inhibits production of T "helper"-2 cytokine IL-10, enhancing T "helper"-1 response. OPN influences cell mediated immunity and enhances B cell immunoglobulin production and proliferation[101].

Apoptosis

In many circumstances OPN acts as an important anti-apoptotic factor. On exposure to harmful stimuli OPN blocks the activation induced cell death of macrophage and T cells as well as fibroblasts and endothelial cells[118, 119]. In inflammatory colitis OPN prevents non programmed cell death[120].

Role in vascular smooth muscle cell (VSMC) remodelling

OPN is a matrix molecule whose expression is increased dramatically by angiotensin II. OPN has been shown to exert an important effect on VSMC growth[121]. OPN induces both formation of new intima after injury and medial thickening without injury suggesting that it plays a role in development of vascular remodelling after angioplasty in vivo. OPN is one of the most highly induced proteins at sites of epithelial injury. In atherosclerotic lesions OPN appears to promote early inflammatory mechanism associated with macrophage recruitment[122].

OSTEOPONTIN: A CULPRIT PROTEIN IN OSTEOARTHRITIS

OPN is considered as a pro inflammatory cytokine. Its altered expression may be involved in pathological conditions viz., inflammatory disease, autoimmune disease, osteoporosis, joint diseases and cancer metastasis[123].

OPN is considered as a critical intrinsic regulator that plays an important role in progression of OA. OPN expression has been shown to be increased in the joints of the patients and it has been reported to be correlated with the severity of inflammatory status and joint lesion in OA patients[124-126]. Honsawek et al[101] analysed OPN levels in both synovial fluid and plasma of patients with OA and healthy subjects and found significantly higher OPN levels in both synovial fluid as well as in plasma of patients with knee OA when compared to healthy subjects. Similarly Mohammed et al[127] has analysed OPN levels in both synovial fluid/ plasma of patients with OA and healthy subjects. Significantly higher OPN levels in both synovial fluid as well as in plasma of patients with knee OA was seen, when compared to healthy subjects. According to these studies the increased level of OPN was suggested to be related with the progressive damage of the joint in OA. Gao et al[125] investigated OPN levels in both articular cartilage and synovial fluid to evaluate the possible correlation with the radiological grading and Mankin score (histo -pathological tool to classify the severity of OA lesions of cartilage). They found higher OPN concentration in articular cartilage and synovial fluid when compared to healthy subjects. Qin et al[128] analysed

synovial fluid OPN in patients with OA and higher OPN mRNA and protein expression was observed and they found it to be closely related to the development of OA. Xu et al[129]analysed the phosphorylation level of OPN in OA cartilage by immuno-precipitation technique. They found a higher phosphorylation level of OPN in OA cartilage when compared to normal cartilage. They suggested that OPN phosphorylation might be related to the cartilage degeneration. Pullig et al[130] analysed the expression of OPN level in OA cartilage in humans and found an increased OPN protein deposition and mRNA expression in deep zone of articular cartilage of patients with OA. Martinez-Calleja et al[131] found that in the early stages of OA in the superficial zone of the articular cartilage there was an increased expression of OPN. Zhang et al. found that in OA patients there was an increased level of OPN in fibroblast-like synoviocytes (FLS). In patients with OA they also investigated the effect of HA on the expression of OPN mRNA of FLS and found that the high expression of OPN mRNA is a consequence of increased HA level in OA synovitis. Mat sui et al[132] generated OPN deficient mice and found that OPN deficiency was associated with more severe OA as OPN might be required for cartilage homeostasis and to prevent OA progression. Thus, these studies suggest that the abnormal level of OPN in synovial fluid, plasma, or articular cartilage might be related to OA and hence **OPN can serve as a valuable diagnostic biomarker for determining the disease severity.**

ROLE OF OSTEOPONTIN IN PATHOGENESIS OF OSTEOARTHRITIS

The increased OPN expression is involved in the molecular pathogenesis of OA, the pathological mechanism being obscure. The main pathological change in OA is the degeneration of articular cartilage[133]. Appropriate joint load stimulates the chondrocytes to maintain the cartilage homeostasis. In chondrocytes abnormal mechanical load alters the composition and metabolism of articular cartilage which then induce the release of OPN and this enhanced level of OPN in cartilage led to the induction of MMP-13[129]. This MMP-13 is thought to be involved in the degradation of cartilage matrix components type II collagen and proteoglycan release from cartilage tissues. The loss of proteoglycan and excessive cleavage of type II collagen facilitated the development of OA[132]. The elevated levels of OPN in cartilage tissue activate the transcription factor NF-κ B pathway which is involved in the production of many inflammatory factors like chemokines and cytokines (IL-1, IL-6, IL-8, CCL2, CXCL1etc) in cartilage that leads to the spontaneous production of these cytokines as well as mediators and exert injurious effects on function of chondrocytes that leads to an imbalance of cartilage homeostasis which results in progressive articular degeneration[125]**.Fig-4.**

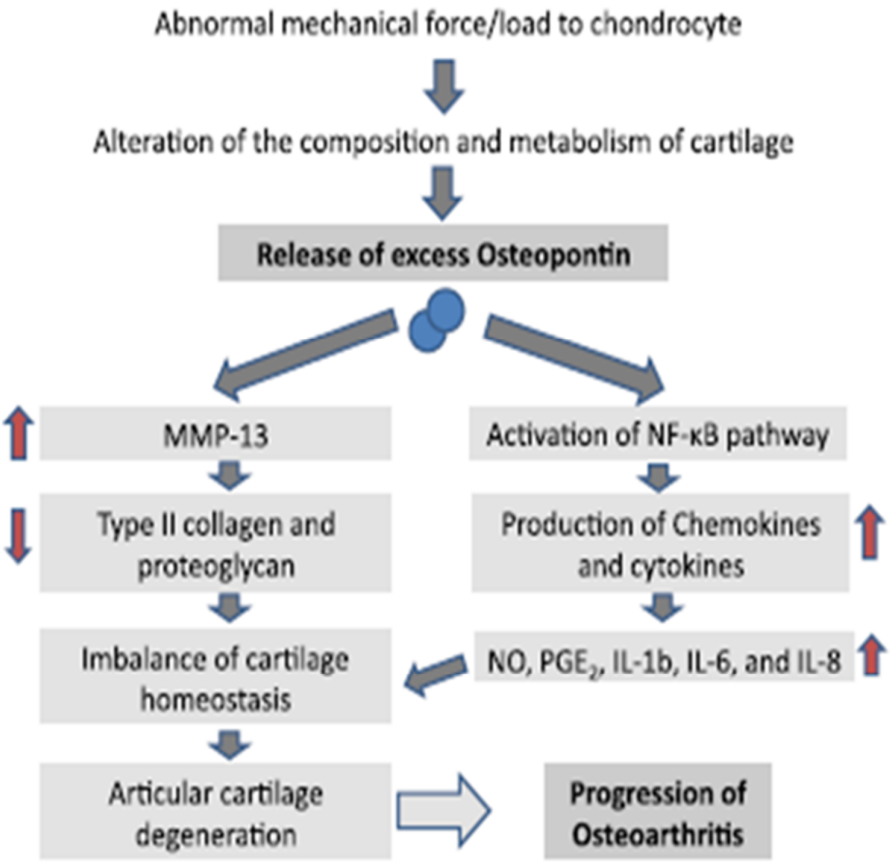


FIGURE-4- ROLE OF OPN IN OA PROGRESSION

DIAGNOSTIC ASPECTS OF OSTEOPONTIN IN OSTEOARTHRITIS

OA, the most common form of arthritis characterized by progressive degeneration of articular cartilage is one of the leading causes of morbidity in the aging population[134]. In the recent times, though the early diagnosis of OA has gained attention with improvement in the prognosis with the conventional therapy, the treatment of OA still remains unsatisfactory. Hence to develop innovative therapeutic strategies, a greater understanding of the pathogenic mechanism of OA becomes essential. For effective prevention and therapeutic treatment of OA, the need for identification of appropriate mediators of joint destruction is required[135]. OPN can serve as a promising biomarker for diagnosis of OA as high level of OPN is frequently found in the synovial fluid and plasma of OA patients.

Singh et al[136] in a recent study proposed stem cells as a therapeutic choice for cartilage repair in OA patients. The use of biochemical markers has recently been proposed to:

i. diagnose the disease at an earlier stage
ii. assess the severity of the disease
iii. to develop a safe and effective disease modifying treatment for OA patients[137].

Hence OPN may serve as a useful diagnostic biomarker for the treatment and prognosis of OA[123].

THERAPEUTIC ASPECTS OF OSTEOPONTIN IN OSTEOARTHRITIS

Therapeutic research on OA may involve two main strategies as follows.

1. To elucidate the mechanism of joint pain
2. The mechanism of articular cartilage degradation[124].

Yumoto et al[138,] in his study to investigate the role of OPN in the pathogenesis of inflammatory process induced a mixture of anti-type II collagen mAbs and lipo polysaccharide (mAbs-LPS) in an arthritis model. They found that OPN deficiency prevents the model mice from loss of proteoglycan in the articular cartilage, swelling and destruction of joints via promotion of angiogenesis and induction of apoptosis in chondrocytes. Yamamoto et al[139] in the murine arthritis model investigated the effect of an anti-OPN antibody (M5Ab) by inducing a mixture of four anti-type II collagen monoclonal antibodies. They found that M5Ab significantly inhibited the cartilage degeneration and suppressed the development of arthritis in their murine study model[139]. Yamamoto et al investigated the effect of C2K1 in CYNOMOLGUS MONKEY by constructing a chimeric anti-OPN antibody and they found that C2K1 prevents the destruction of bone and cartilage in the joints of this collagen induced arthritis in non-human primates significantly.

Fan et al[140] in his study reported that anti-OPN mAb, 23C3 reduces the levels of serum type II collagen specific autoantibodies and pro inflammatory cytokines and suppressed T cell recall responses to type II collagen and inhibits

the development of collagen induced arthritis in DBA/1J mice immunized with type II collagen to induce collagen induced arthritis[140].

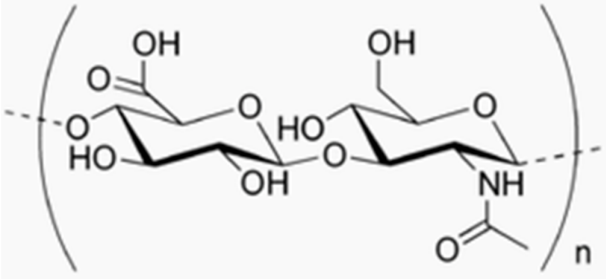
Due to lack of enough evidence more investigations are necessary to define the signalling events induced by OPN for the inhibition of OPN mediated inflammatory process, because targeting these signalling events and reducing the expression of OPN, may be successful in the treatment of OA.

HYALURONIC ACID

Hyaluronic acid (HA) is an anionic, non-sulphated glycosaminoglycan present in connective tissue, neural and epithelial tissues. This high molecular weight glycosaminoglycan is formed in the plasma membrane and is one of the chief components of ECM and contributes to cell proliferation and migration as seen in progression of some malignant tumours[144, 145]. The average 70 kg man has 15gms of hyaluronic acid in the body, one-third of which is synthesized and degraded daily[146].

Structure

Structure of Hyaluronic acid



In the 1930s Karl Meyer first determined the properties of hyaluronic acid[147]. Hyaluronic acid is a polymer of disaccharides composed of

D-N-acetyl glucosamine and D- glucuronic acid linked by alternating β-1,4 and β-1,3 glycosidic bonds. In human synovial fluid, the average molecular weight of hyaluronic acid is 3-4 million Da and polymers can range in size from 5000-20,000,000 Da in vivo. Hyaluronic acid can be 25,000 disaccharide repeats in length.

Synthesis

HA is synthesized by a class of integral proteins namely hyaluronan synthase of three types - HAS1, HAS2, HAS3. These enzymes lengthen HA by adding N-acetyl glucosamine and glucuronic acid repeatedly to the nascent polysaccharide as it is extruded via ABC transporter through the cell membrane into the extracellular space[148].

HA synthesis has been shown to be inhibited by a 7-hydroxy 4-methyl coumarin derivative which is 4-methyl umbelliferone[149]. This selective inhibition is helpful in preventing the metastasis by tumour cells[150]. When tested in cultured human synovial fibroblasts, there is a feedback inhibition of HA synthesis by low molecular weight HA (<500kDa) at high concentrations and stimulation by high molecular weight HA (>500kDa) [151].

Functions

Until late 1970s HA was thought to be a major component of the synovial fluid which increased the viscosity of the fluid and had lubricating function along with lubricin. HA is an important component of articular cartilage which coats around each chondrocyte. Aggrecan monomers bind to

hyaluronan in the presence of hyaluronan and proteoglycan link protein 1 (HAPLN1) forming large negatively charged aggregates that imbibe water contributing to the resilience of the cartilage. The amount of HA in cartilage increases but the molecular weight of HA decreases with age[152].

HA is a major component of skin and is involved in tissue repair. HA degradation products accumulate in the skin after UV exposure because the cells in the dermis stop producing HA thereby increasing its rate of degradation[153].

HA contributes to proliferation and migration of cells. It participates in a number of cell surface receptor interactions. Its contribution to tumour growth is due to its interaction with CD44 which is required for cell adhesion interactions of tumour cells.

Degradation

HA is degraded by hyaluronidases. In humans, there are seven types, most of which are tumour suppressors. HA on degradation forms oligosaccharides and very low molecular weight HA which exhibit pro-angiogenic properties[154] and they can also induce inflammatory responses in macrophages and dendritic cells in tissue injury[155, 156].

Cell receptors for HA

Cell receptors for HA fall into three groups- CD44, receptor for HA mediated motility (RHAMM) and intercellular adhesion molecule-1(ICAM-1).

The binding of HA-CD44 was first demonstrated by Aruffo et al[157] in 1990 and till date it is recognized as the main cell surface receptor for HA. This binding plays an important role in the various physiologic events viz., cell migration, proliferation, activation, cell-cell and cell-substrate adhesion, endocytosis of HA leading to its catabolism in macrophages and assembly of peri-cellular matrices from HA and proteoglycan[158, 159].

ICAM-1 is a metabolic cell surface receptor for HA and is responsible mainly for the clearance of HA from lymph and blood plasma[158, 160]. Ligand binding of this receptor triggers a highly co-ordinated cascade of events that include the formation of endocytic vesicle and its fusion with primary lysosomes which causes enzymatic digestion to monosaccharides which are transported to cell sap, phosphorylation of GlcNac and enzymatic deacetylation[158,161,162]. The binding of HA to ICAM-1 mediated inflammatory activation.[159]

CLINICAL SIGNIFICANCE

HA levels correlate well with malignancy. Hence it is often used as a tumour marker (prostate and breast cancer), and to monitor the prognosis of the disease[163, 164].

Wound repair and inflammation

In the early inflammatory phase of wound repair there is abundant HA in wound tissue due to increased synthesis[159]. HA acts as a promoter of early

inflammation and acts as a moderator of inflammatory response and helps in stabilization of granulation tissue matrix. HA can protect against free radical damage in cells[165]. HA functions in the negative feedback loop of inflammatory activation[159]. TNF-α stimulates the expression of TSG-6(TNF-stimulated gene 6), a HA binding protein which forms a stable complex with serum proteinase inhibitor IαI (Inter-α-inhibitor) with a synergistic effect on latters plasmin inhibitory activity. Plasmin is involved in the activation of proteolysis cascade of matrix metalloproteinases and other proteinases causing inflammatory tissue damage. Hence the action of TSG-6/IαI complex may additionally get organised by binding to HA in the ECM and may serve as a potent negative feedback loop to inflammation and stabilize the granulation tissue as healing progresses[159, 166].

Granulation and organization of the granulation tissue matrix

HA is abundant in the granulation tissue matrix and are essential for tissue repair. HA helps in cell migration, proliferation, and organization of granulation tissue matrix[159]. HA is synthesized in the plasma membrane and released directly into the extracellular environment, contributing to the hydrated microenvironment at the sites of synthesis. In the basal layer of the epidermis where proliferating keratinocytes are found there is a relative high concentration of HA in normal skin[167]. The main functions of HA in epidermis are maintaining the extracellular space and providing an open, well hydrated structure for the passage of nutrients.

Foetal wound healing and scarring

HA content in foetal wounds is higher than in adults suggesting that HA atleast in part may reduce collagen deposition and hence may lead to reduced[168] scarring.

HYALURONIC ACID IN OSTEOARTHRITIS

Even though majority of circulating HA originates from extra cartilaginous sources, it is considered as a marker of cartilage degradation which can be detected in the serum and synovial fluid[98]. **The proteoglycan reflects cartilage turnover whereas HA reflects synovial activity[98].** In addition, higher serum HA levels have been correlated with the number of joints involved and degree of clinical disability. These findings support the theory that serum HA levels reflect synovial hyperactivity. Hence serum HA levels may serve as a predictor of OA disease progression[98] to be correlated with the radiological evidence of disease progression over a five year interval, as patients with advanced disease had higher levels of HA than at the outset[98].

HA is produced locally by cells of the ECM. In addition to its structural properties it is thought to play a role in cell signalling. The release of HA/HA fragments into the systemic circulation is due to degradation and turnover of the ECM from arthritic joints. After entering the systemic circulation they are cleared by the liver as well as the lymphatics via a HA-specific receptor after endocytosis[141]. Increased production and release of HA are thought to reflect the localized inflammation occurring in the synovial lining and the cartilage

degradation[142]. The joint stiffness and oedema in arthritis is due to increased HA in inflamed synovium[143].

Accordingly in this case control study, the concentrations of plasma OPN and serum HA in patients with knee OA was assessed at Rajiv Gandhi Government General Hospital to evaluate the diagnostic value of plasma OPN and serum HA in patients with knee OA.

Aims & objectives

AIMS & OBECTIVES

AIMS:

1. To evaluate, if plasma osteopontin can be used as a biomarker to monitor the severity of knee osteoarthritis.
2. To assess the role of osteopontin in the pathogenesis of osteoarthritis by stimulating MMP13, thereby increasing hyaluronic acid levels in serum.

OBJECTIVES:

1. To correlate plasma osteopontin level with radiological grade in patients with knee osteoarthritis.
2. To correlate plasma osteopontin level with serum hyaluronic acid in patients with knee osteoarthritis.

Materials & methods

MATERIALS AND METHODS

The present study - "Correlation of plasma osteopontin with radiological grading in patients with osteoarthritis in the knee joint", is a case control study carried out during the period between January 2015 to June 2015 at Madras Medical College and Rajiv Gandhi Government General Hospital. This study was conducted after obtaining **INSTITUTIONAL ETHICS COMMITTEE** clearance.

Population of the study

Inclusion criteria for cases

The study group comprised of 60 cases of osteoarthritis patients, comprising of 48 adult females and 12 adult males. Patients with signs and symptoms as well as radiologic evidence of OA were selected. Patients were on treatment with NSAIDs and calcium supplements. The inclusion criteria for cases include:

1. Unilateral or bilateral knee osteoarthritis
2. Duration of knee pain > three months
3. Age 40-70 yrs
4. Radiological evidence of osteoarthritis

Inclusion criteria of control

The control group comprised of 30 adults, age and gender matched individuals, with 23 females and 7 males. They had no symptoms and signs of OA and their X-ray knee joint was normal- no evidence of OA.

Exclusion Criteria

1. Patients with secondary osteoarthritis-post-traumatic or post inflammatory
2. Renal failure & hepatic failure patients
3. Generalised osteoarthritis
4. Patients with both hip and knee osteoarthritis
5. Patients with malignancy

Sample collection and storage

After obtaining informed consent from the patients attending the Out-Patient Department, Institute of Orthopaedics, Rajiv Gandhi Government General Hospital, Chennai, blood samples were collected.

5mL of blood was collected from the ante-cubital vein of the study population- both cases and controls (60,30 respectively). The blood was aliquoted in to three tubes. One tube containing EDTA as anticoagulant and 2mL of blood was transferred up to the prescribed mark on the tube. The tube was gently shaken between the palms for uniform mixing. In another red capped serum tube another 2mL of blood was transferred. The remaining 1mL of the blood was transferred in to a plain tube for the further processing of the sample.

The sample tubes were labelled and stored in ice box during their transport to the biochemistry laboratory. The samples were centrifuged within

45 minutes of collection and plasma/serum from each tube was aliquoted into a separate sample storage eppendorf tubes. Samples were stored in deep freezer at-20^{o}C until further processing. **Plasma samples were used for osteopontin estimation and serum samples were used to estimate hyaluronic acid, calcium and phosphorus**.

ESTIMATION OF PLASMA OSTEOPONTIN

Plasma osteopontin level was estimated using the Sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) method (Ray Biotech). The kit was stored at 2-8^{o}C in a refrigerator.

Principle

Quantitative ELISA using sandwich technique was performed to analyse OPN concentration in the plasma of both cases and controls. The micro-plate provided was already coated with an antibody specific for human OPN. The standards and samples were then pipetted into the wells and the OPN present in the sample were bound to the wells by the immobilized antibody. The wells were then washed and biotinylated anti-human OPN antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted in to the wells. The wells were then again washed, and a TETRAMETHYLBENZIDINE (TMB) substrate solution was added to the wells and colour developed in proportion to the amount of OPN bound. The stop solution was then added and the colour changed from blue to yellow, and the intensity of the colour was then measured at 450 nm.

Reagents

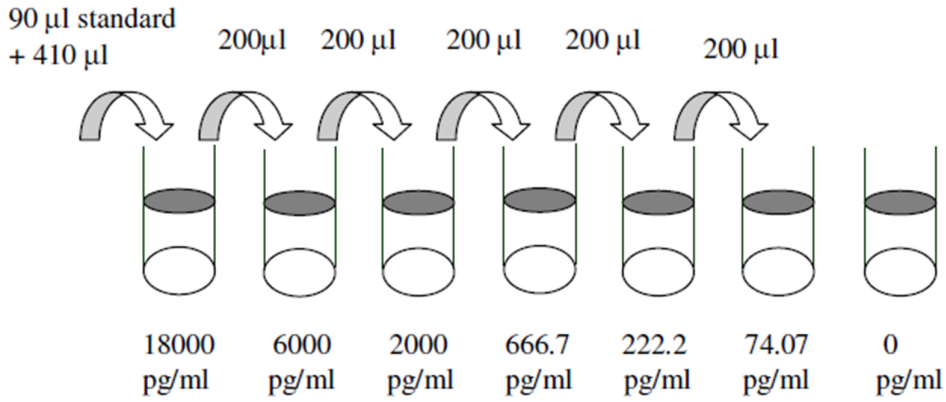
1. OPN micro plate: 96 wells (12 strips x 8 wells) coated with anti-human OPN.
2. 25 mL of 20 x wash concentrate solution.
3. 2 vials of recombinant human OPN standards.
4. 30 mL of animal serum with 0.09% sodium azide as preservative (Assay Diluent A) was used as standard and sample diluent for serum and plasma samples.
5. 15 mL of 5x concentrated buffer (Assay Diluent B) can be used as standard and sample diluent for culture and urine samples.
6. Biotinylated anti-human OPN vials were used as detection antibody OPN.
7. 200 µL of 500 x concentrated HRP-conjugated streptavidin (HRP-Streptavidin Concentrate).
8. 12 mL of 3,3',5,5'- tetramethylbenzidine (TMB) in buffered solution was used as one step TMB substrate solution.
9. 8 mL of 0.2 M sulfuric acid was used as stop solution.

Preparation of reagents and standards:

All reagents and samples were brought to room temperature (18 - 25°C) before use.

1. Sample dilution: Samples were diluted (1in100 dilution) with Assay Diluent A.

2. Assay Diluent B was diluted to 5-fold with deionized water.
3. Standard preparation: First the vial containing standard was given a brief spin before preparing various concentrations of the standard and for plasma samples 400 µL Assay Diluent A was added into standard vial to prepare a 100 ng/mL standard. Then the powder was dissolved thoroughly by a gentle mix. 90 µL of OPN standard from the vial was added into a tube with 410 µL Assay Diluent A to prepare 18,000 pg/mL stock solution of the standard. Finally 400 µl of Assay Diluent A was pipetted into each tube. Using the stock standard solution, dilution series as shown below was prepared. Each tube was thoroughly mixed before the next transfer. Assay Diluent A served as the zero standard (0 pg/mL).



4. The (20x) wash concentrate was checked for any visible crystals and mixed gently until dissolved. To 20 mL of wash buffer concentrate 380 mL of deionized water was added to prepare 400 mL of 1x wash buffer.

5. The Detection Antibody vial was given a brief spin before use. 100 µL of 1x Assay Diluent B was then added into the vial to prepare a detection antibody concentrate. Gentle mixing was done by pipetting up and down before use. The detection antibody concentrate was then diluted to 80-fold with 1x Assay Diluent B.
6. After a brief spin of the HRP-Streptavidin concentrate vial gentle mixing was done by pipetting up and down before use. HRP Streptavidin concentrate was then diluted to 500-fold with 1x Assay Diluent B.

Procedure:

1. All reagents and samples were brought to room temperature (18 - 25℃) before use.
2. 100 µL of each standard and sample was added into appropriate wells and incubated for 2.5 hours at room temperature.
3. The solution was then discarded and washed 4 times with 1x Wash Solution (200 µL each).
4. 100 µL of 1x prepared biotinylated antibody was then added to each well and incubated for 1 hour at room temperature.
5. The solution was discarded and then washed 4 times with 200µL of 1x Wash Solution for each well.
6. 100 µL of prepared Streptavidin solution was then added to each well and incubated for 45 minutes at room temperature.

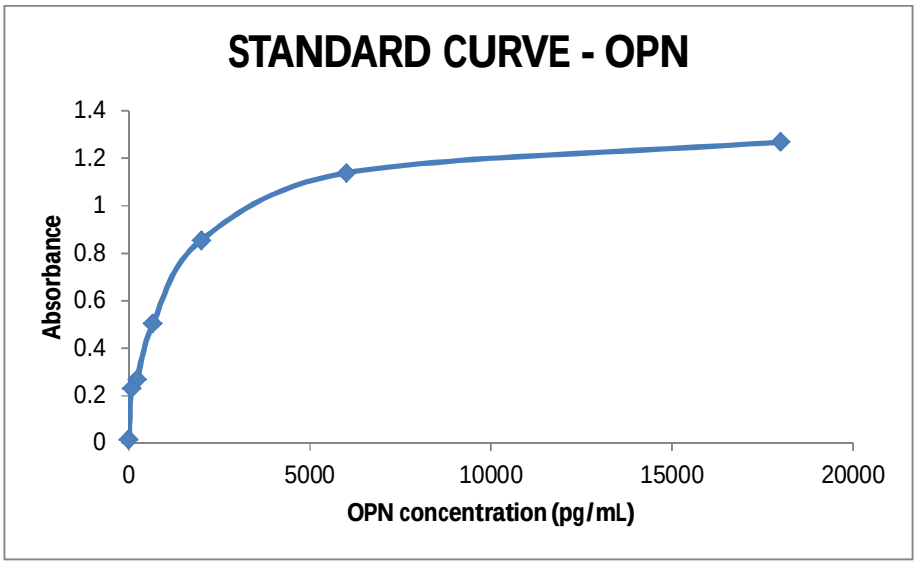
7. The solution was then discarded and washed 5 times with 200µL of 1x Wash Solution for each well.
8. 100 µL of TMB one-step substrate reagent was then added to each well and incubated for 30 minutes at room temperature.
9. 50 µL of Stop Solution was then added to each well and read at 450 nm immediately.

Calculation of results

The standard and sample optical density was subtracted from the zero standard optical density.

The standard curve was plotted with standard concentration on the x-axis and absorbance on the y-axis and the best-fit straight line was drawn through the standard points**.** The concentration of plasma osteopontin in each sample was determined from the standard graph.

STANDARD CONCENTRATION (pg/mL)	**ABSORBANCE**
18000	1.269
6000	1.1393
2000	0.8548
666.7	0.5041
222.2	0.2698
74.07	0.2312
0	0.0142



Sensitivity

The minimum concentration of OPN detected by the kit was < 50 pg/mL

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Specificity

Cross Reactivity: This ELISA kit shows no cross-reactivity with other cytokines.

Linearity: up to 18000pg/mL

ESTIMATION OF SERUM HYALURONIC ACID

The serum hyaluronic acid level was estimated using the Sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) method (Cusabio). The kit was stored at 2-8^{o}C in a refrigerator.

Principle:

Quantitative sandwich enzyme immunoassay technique was employed for estimation of serum HA. Antibody specific for HA were pre-coated onto a

micro plate. The standards and samples were then pipetted into the wells and any HA present in the sample was bound by the immobilized antibody. The wells were then washed to remove any unbound substances, then a biotin-conjugated antibody specific for HA was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of HA bound in the initial step. The colour development was then stopped by using a stop solution and the intensity of the colour was measured.

Reagents:

1. Micro plate for assay (12×8 strips coated micro wells).
2. 2 vials of freeze dried standard.
3. 120µL of 100x concentrate of Biotin- antibody
4. 120µL of 100x concentrate of HRP- avidin
5. 15 mL of Biotin-antibody diluent
6. 15mL of HRP- avidin diluent
7. 50mL of Sample diluent
8. 20mL of 25 x concentrate Wash buffer
9. 10mL of TMB substrate solution
10. 10 mL of stop solution

The serum samples were diluted with sample diluent (1:100) before test. The suggested 100-fold dilution was achieved by adding 10µL sample to 40µL

of sample diluent. The 100-fold dilution was completed by adding 15µL of this solution to 285µL of Sample Diluent.

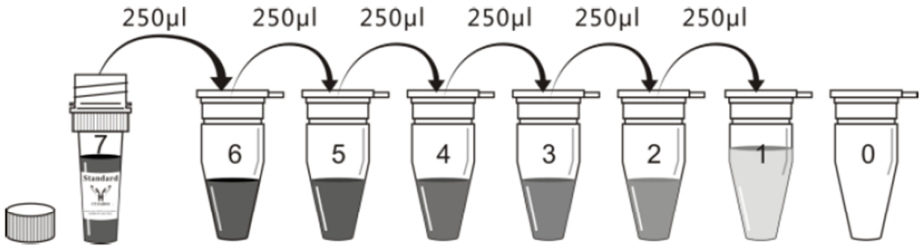
Preparation of reagents and standards:

Bring all reagents to room temperature (18-25°C) before use.

1. Biotin-antibody (1x) - The vial was centrifuged before opening. Biotin-antibody required a 100-fold dilution. The100-fold dilution was made by adding 10 µL of Biotin-antibody with 990 µL of Biotin-antibody Diluent.
2. HRP-avidin (1x) - The vial was centrifuged before opening. HRP-avidin required a 100-fold dilution. The100-fold dilution was prepared by adding10µL of HRP-avidin to 990µL of HRP-avidin Diluent.
3. Wash Buffer(1x)- The wash buffer was checked for the presence of crystals before use, kept at room temperature and mixed gently until the crystals have completely dissolved. To 20 mL of (25 x) Wash Buffer Concentrate 480mL of deionized water was added to prepare 500 mL of (1x) Wash Buffer.
4. Preparation of standards - The standard vial was centrifuged at 6000-10,000 rpm for 30s before use.

The standard was reconstituted with 1.0 mL of Sample diluent. This reconstitution provided a stock solution of 10ng/mL. Then the standard was mixed to ensure complete reconstitution and the standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

250µL of sample diluent was then pipetted into each tube (S0-S6). The stock solution was used to produce a 2- fold dilution series. Each tube was thoroughly mixed before the transfer. The undiluted standard served as the high standard (10 ng/mL). The sample diluent served as the zero standard (0 ng/mL).



Tube	S7	S6	S5	S4	S3	S2	S1	S0
ng/ml	10	5	2.5	1.25	0.625	0.312	0.156	0

Procedure:

All reagents and samples were brought to room temperature before use.

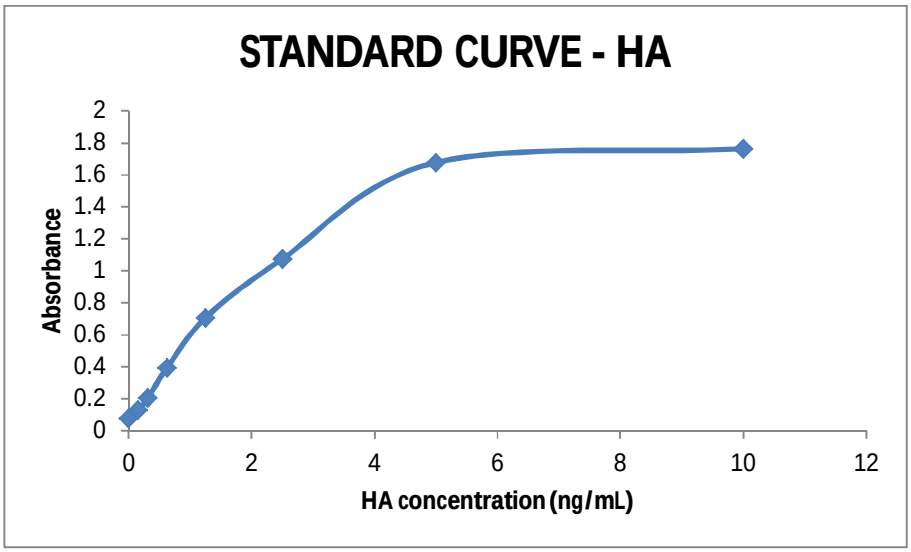
1. 100µL of standard and sample were added per well and then incubated at 37℃ for 2 hours.
2. The liquid of each well was removed without washing .
3. 100µL of Biotin-antibody (1x) was then added to each well and incubated at 37℃ for 1 hour.
5. With ELISA washer three washes was performed with Wash Buffer 200µL for each well. After the last wash, the remaining wash Buffer was removed by decanting on a filter paper.

6. 100µL of HRP-avidin (1x) was then added to each well and incubated at 37°C for 1 hour.
7. The wash process was repeated for five times as in step 5.
8. 90µL of TMB Substrate was added to each well and incubated at 37°C for 15-30 minutes.
9. 50µL of stop solution was added to each well, the plate was gently tapped to ensure thorough mixing.
10. The optical density of each well was determined within 5 minutes, using a micro plate reader set at 450 nm.

Calculation of results

A standard curve was constructed by plotting the absorbance for each standard on the y-axis against the concentration on the x-axis and a best fit curve through the points on the graph was drawn. The data may be linearized by plotting the log of the HA concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis.

STANDARD CONCENTRATION (ng/mL)	ABSORBANCE
10	1.7628
5	1.6773
2.5	1.0754
1.25	0.7067
0.625	0.3947
0.312	0.2056
0.156	0.1291
0	0.0779



Detection range - 0.156ng/mL - 10 ng/mL.

Sensitivity

The minimum concentration of human HA that can be detected by the kit is < 0.039ng/mL.

The sensitivity of the assay, or LOWER LIMIT of DETECTION (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined the mean O.D value of 20 replicates of the zero standard added by their three standard deviations.

Specificity

This assay has high sensitivity and excellent specificity for detection of human HA. No significant cross-reactivity or interference between human HA and analogues was observed.

ESTIMATION OF SERUM CALCIUM AND PHOSPHORUS

The estimation of serum calcium and phosphorus was done to know the relationship of these parameters besides the radiological grades in patients with knee OA and to rule out secondary causes of OA.

Estimation of serum calcium

Serum calcium was estimated in the samples using Accucare Calcium Arsenazo III method in MERCK semi autoanalyzer.

Principle:

At neutral pH, calcium reacts with Arsenazo III (1,8- dihydroxy-3,6-disulpho-2,7-naphthalene-bis(azo)-dibenzenearsonic acid), it forms a blue coloured complex. The intensity of the colour formed is proportional to the calcium concentration in the sample.

Reagent Composition:

Reagent 1 - Arsenazo III reagent

Calcium standard - 10 mg/dL

Method:

Arsenazo III method, End point assay.

Assay Procedure:

The assay was performed after calibration. The standard used was 10mg/dL. 25 µL of the sample was added to 1000 µL of the reagent, mixed well and incubated at room temperature for 5 min. Final absorbance of the

sample (Ac) and standard (As) against the reagent blank was measured at 650 nm.

Calculation:

$$\text{Serum/Plasma} = \frac{\text{(Ac) sample}}{\text{(As) standard}} \times \text{Standard concentration}$$

Linearity:

The kit is linear up to 16 mg/dL.

Reference Values:

Serum: 8.8 – 10.2 mg/dL

Estimation of phosphorus

Serum phosphorus was estimated in the samples using pathozyme phosphorus molybdate U.V. method in MERCK semi autoanalyzer.

Principle:

When inorganic phosphorus reacts with ammonium molybdate in an acidic medium it forms a phosphomolybdate complex, which can be measured in the U.V. range at 340 nm. The absorbance of this complex is directly proportional to the amount of inorganic phosphorus present in the sample.

Reagent Composition:

Molybdate Reagent

Phosphorus standard: 5 mg/dL

Method:

Molybdate U.V method, End point assay

Procedure:

The assay was performed after calibration. The standard used was 5mg/dL. 10 µL of the sample was added to 1000 µL of the reagent, mixed well and incubated for 5 min at room temperature and the absorbance of the standard (Abs.S) and the test sample (Abs.T) against the reagent blank (Abs.B) was measured at 340 nm.

Reference Values:

Serum Adults : 2.5 – 5.0 mg/dL

Serum Children : 4.0 – 6.5 mg/dL

Calculation:

$$\text{Phosphorus in mg/dL} = \frac{(\text{Abs.T})-(\text{Abs.B})}{(\text{Abs.S})-(\text{Abs.B})} \times 5$$

Linearity:

The kit is linear upto 20 mg/dL.

Statistical analysis

STATISTICAL ANALYSIS

Statistical analysis was performed for the present study using the "statistical package for social sciences (SPSS) software". Student's t-test was performed for comparison between groups. Pearson's correlation coefficient was employed to determine the correlation among the concentration of plasma osteopontin and serum hyaluronic acid and the Kellgren-Lawrence (K/L) grades. **P-values <0.05** were considered statistically significant.

1. Continuous variables age, height, weight, BMI, the concentrations of calcium, phosphorus, OPN & HA in cases and controls were compared by student "t" test.
2. The gender distribution between cases and controls was compared using chi-square test.
3. OPN level with various Kellgren-Lawrence(K/L) grades of OA was compared using ANOVA.
4. HA level with various K/L grades of OA was compared using ANOVA.
5. Pearson correlation was used to measure the strength of relationship between OPN, HA and K/L grading.
6. Pearson correlation was used to assess the relationship between OPN and HA.
7. ROC curve was used to analyse the diagnostic value of OPN and HA for OA.

Results

MASTER CHART OF PATIENTS WITH OA KNEE

CASES	Age in years	Sex	Height (cm)	Weight (kg)	BMI (kg/m2)	SBP (mmHg)	DBP (mmHg)	K/L grade	OPN (pg/mL)	HA (ng/mL)	Calcium (mg/dL)	Phosphorus (mg/dL)
1	52	Female	150	65	28.88	120	70	3	770.6	2.434	9.3	3.7
2	60	Female	155	60	25	120	70	3	808.2	2.525	9.8	4.3
3	38	Female	152	65	28.13	110	70	2	790.2	2.429	9.1	3.1
4	62	Female	155	62	25.83	140	80	4	4090	3.346	9.2	4.5
5	60	Female	150	75	33.33	130	80	3	990.4	2.371	9.3	3.6
6	54	Female	154	70	29.53	130	80	3	1462	2.419	9.5	4.4
7	52	Female	154	85	35.86	130	80	3	1489	1.75	9.4	4.1
8	50	Female	154	60	25.31	100	70	2	570	2.27	9.7	3.7
9	55	Female	151	87	38.16	130	80	3	1490	2.31	9.2	4.3
10	65	Female	150	55	24.44	110	70	3	1120	2.513	9.5	4.4
11	51	Female	154	70	29.53	110	70	4	1532	3.388	9.4	4.3
12	40	Female	152	65	28.13	100	60	3	522.5	3.471	9.3	4.4
13	56	Female	150	55	24.44	120	70	4	1038	1.921	9.8	4.8
14	63	Female	150	65	28.88	130	80	2	410.4	2.215	9.6	4.4
15	40	Female	152	55	23.8	110	70	3	1104	0.897	9.2	4.1
16	48	Female	152	55	23.8	100	60	3	490.8	3.136	9.9	4.3
17	58	Female	150	60	26.66	110	70	4	344.8	2.844	9.5	4.2
18	65	Female	150	50	22.22	100	70	4	855	2.389	9.6	4
19	53	Female	148	55	25.11	120	70	3	919.1	2.539	9.8	4.3
20	60	Female	154	55	23.2	120	70	4	787.3	3.056	9.4	4.5
21	57	Female	148	52	23.74	130	70	2	643.2	1.744	9.1	4.3
22	51	Female	155	59	24.58	140	76	2	521.1	1.221	9.6	4.1
23	65	Female	153	67	28.63	110	74	3	372.3	2.518	9.7	4.4
24	55	Female	155	62	25.83	130	74	3	961.1	2.297	9.2	3.6
25	50	Female	151	75	32.89	120	70	3	657.4	2.455	9.4	3.5
26	75	Female	145	50	23.8	130	80	3	602.9	2.169	9.5	3.9
27	60	Female	149	50	22.52	110	70	4	1847	3.333	9.2	4.1
28	45	Female	151	52	22.8	100	70	2	555.3	1.813	9.8	3.6
29	55	Female	154	50	21.09	130	84	3	745.3	3.16	9.1	3.9
30	53	Female	156	57	23.45	110	74	4	747.1	2.746	9.5	4.5

CASES	Age in years	Sex	Height	Weight	BMI	SBP	DBP	K/L grade	OPN	HA	Calcium	Phosphorus
			(cm)	(kg)	(kg/m2)	(mmHg)	(mmHg)		(pg/mL)	(ng/mL)	(mg/dL)	(mg/dL)
31	52	Female	154	55	23.2	140	80	3	651.7	2.274	9.7	3.5
32	53	Female	160	75	29.29	120	74	3	455.5	5.31	9.4	3.6
33	55	Female	153	85	36.32	130	84	3	605.1	2.388	9.6	3.7
34	50	Female	152	54	23.37	120	74	4	887.1	3.068	9.3	4.5
35	40	Female	149	58	26.12	100	70	4	1779	2.855	9.7	4.1
36	60	Female	151	59	25.87	130	80	3	932.1	4.125	9.5	4.2
37	55	Female	152	65	28.13	110	74	3	574	3.664	9.8	4
38	60	Female	150	50	22.22	130	80	3	780.4	1.05	9.6	4.2
39	50	Female	155	65	28.08	120	74	3	471.8	2.43	9.2	4
40	47	Female	151	60	26.31	130	74	3	260.5	4.489	9.3	4.2
41	50	Female	156	59	24.27	110	74	3	805	1.832	9.1	4.1
42	60	Female	153	67	28.63	100	70	3	1478	1.444	9.6	4.5
43	65	Female	155	57	23.75	120	74	3	964.2	2.523	9.4	4.4
44	53	Female	153	53	22.64	120	74	3	888.3	3.601	9.5	3.7
45	63	Female	156	66	27.16	130	74	3	622.6	4.815	9.3	3.7
46	65	Male	168	82	29.07	140	80	2	531.1	1.882	9.6	3.9
47	62	Male	164	66	24.53	130	80	4	929.3	3.377	9.4	3.6
48	73	Male	165	69	25.36	120	84	3	581	3.442	9.6	4.5
49	70	Female	152	58	25.1	120	70	2	517.2	0.942	9.5	4.2
50	55	Male	162	53	20.22	130	80	2	487.9	1.16	9.3	3.9
51	53	Male	157	74	30.08	130	86	3	530.1	2.064	9.4	3.5
52	57	Male	165	60	22.05	140	80	3	1294	2.231	9.7	3.6
53	65	Male	172	75	25.33	130	84	3	4541	4.16	9.3	3.8
54	59	Male	168	72	25.53	120	70	3	1037	2.704	9.5	3.7
55	65	Male	165	80	29.41	130	80	3	441.2	1.961	9.2	4.1
56	60	Male	169	68	23.85	110	74	4	749.2	3.109	9.6	3.7
57	56	Male	172	73	24.74	140	80	3	738.8	1.463	9.2	3.6
58	60	Male	166	64	23.27	120	74	3	705.9	1.902	9.7	4.5
59	70	Female	160	55	21.48	130	80	4	986.7	2.768	9.6	3.8
60	65	Female	158	52	20.88	120	74	4	3632	2.263	9.4	4

MASTER CHART OF CONTROLS

CONTROLS	Age in	Sex	Height	Weight	BMI	SBP	DBP	K/L grade	OPN	HA	Calcium	Phosphorus
	years		(cm)	(kg)	(kg/m2)	(mmHg)	(mmHg)		(pg/mL)	(ng/mL)	(mg/dL)	(mg/dL)
1	61	Female	162	53	20.15	110	74	.	783.7	2.665	9.6	4.3
2	60	Female	150	52	23.11	100	70	.	540.1	1.245	9.4	3.7
3	65	Female	155	60	25	120	70	.	503.1	1.57	9.9	3.9
4	55	Female	156	63	25.92	130	80	.	658.2	2.959	9.5	4.2
5	60	Female	164	56	20.82	110	70	.	541.6	2.062	9.3	3.8
6	48	Female	159	55	21.74	100	70	.	532.1	1.682	9.2	3.9
7	55	Female	161	60	23.16	120	74	.	655.9	2.224	9.8	3.6
8	56	Female	160	58	22.65	110	70	.	596.5	1.983	9.9	4.3
9	38	Female	158	52	20.88	130	80	.	596.5	1.948	9.7	3.6
10	54	Female	162	54	20.61	120	70	.	613.3	1.151	9.6	3.8
11	63	Female	159	58	22.92	100	70	.	739.9	1.484	9.3	4.3
12	55	Female	161	64	24.71	110	70	.	397.3	1.767	9.4	3.7
13	45	Female	164	60	22.3	120	80	.	607.7	1.75	9.3	4.4
14	50	Female	162	55	20.99	130	70	.	770.1	1.681	9.5	3.6
15	55	Male	168	58	20.56	120	74	.	576.7	1.586	9.6	3.8
16	65	Male	170	64	22.14	140	80	.	983.7	2.204	9.1	4.4
17	51	Female	157	58	23.51	110	74	.	980.3	2.279	9.7	4.2
18	70	Female	162	61	23.38	130	80	.	522.2	2.514	9.2	3.8
19	65	Female	164	58	21.64	120	74	.	923.5	1.806	9.3	3.6
20	60	Female	156	62	25.51	100	70	.	607	1.547	9.4	4.1
21	40	Female	164	60	22.38	120	70	.	250.6	0.854	9.6	3.9
22	53	Male	168	68	24.11	130	74	.	500.5	2.271	9.8	4.2
23	62	Male	166	62	22.54	110	70	.	519.4	1.239	9.9	4
24	57	Male	168	64	22.69	130	80	.	919.2	2.024	9.5	3.6
25	75	Female	160	56	21.87	120	70	.	466.8	2.528	9.9	4.1
26	47	Female	163	62	23.39	110	80	.	990.9	1.461	9.3	4.4
27	53	Female	162	57	21.75	100	70	.	655.5	2.369	9.6	4.2
28	50	Female	158	60	24.09	130	80	.	248.9	2.018	9.5	3.7
29	60	Male	164	65	24.25	120	74	.	398.7	1.56	9.8	4.3
30	72	Male	169	68	23.86	140	80	.	251.6	1.841	9.6	3.6

RESULTS

TABLE – 1

Age distribution of patients among cases (OA knee) and controls

	Group	N	Mean	Std. Deviation	P value
Age in years	Control	30	56.67	8.683	0.971
	Cases	60	56.60	7.907	

In the present study 60 patients with varying grades of OA, confirmed by X-ray knee joint and 30 controls, were enrolled. The mean and standard deviation of OA patients and controls were 56.60+/-7.907 and 56.67+/-8.683 respectively. There was no statistical difference in the age between OA patients and controls (p=0.971).

TABLE-2

Gender distribution among patients with OA knee and controls

	Controls	Cases	p-value
Males	7	12	0.715
	36.8%	63.2%	
Females	23	48	
	32.4%	67.6%	

The above gender distribution among patients with OA knee and controls show that females constitute higher percentage than males in both cases and controls. There is no significant difference in gender among cases and controls (p=0.715).

Comparison of patient characteristics

TABLE - 3

	Group	N	Mean	Std. Deviation	P value
Height	Control	30	161.73	4.571	0.123
	Cases	60	155.28	6.325	
Weight	Control	30	59.43	4.305	**<0.001****
	Cases	60	62.87	9.675	
BMI	Control	30	22.7543	1.51611	**<0.001****
	Cases	60	26.0972	3.77687	

** HIGHLY SIGNIFICANT

TABLE - 4

	Group	N	Mean	Std. Deviation	P value
SBP	Control	30	118.00	11.861	0.234
	Cases	60	121.17	11.802	
DBP	Control	30	73.93	4.346	0.458
	Cases	60	74.80	5.575	

Table-3 & Table-4 compares the mean, standard deviation and p-value of cases and controls with respect to variables height, weight, BMI, systolic and diastolic BP. There is a highly statistically significant difference with respect to weight and BMI ($p=<0.001^{**)}$ among cases and controls, while there is no significant difference among cases and controls with respect to height, systolic and diastolic BP.

TABLE – 5

Comparison of calcium and phosphorus levels among cases and controls

	Group	N	Mean	Std. Deviation	P value
Calcium	Control	30	9.540	0.2328	0.105
	Cases	60	9.460	0.2109	
Phosphorus	Control	30	3.967	0.2881	0.365
	Cases	60	4.035	0.3569	

Table-5 shows the mean, standard deviation and p-value of the analytes - calcium and phosphorus. The mean and standard deviation of calcium and phosphorus of patients with OA knee and controls are 9.46+/-.21,9.54+/-.23 and 4.03+/-.35,3.97+/-.28 respectively. There is no statistically significant difference among cases and controls with respect to calcium and phosphorus (p-value .105 and .365 respectively).

TABLE – 6
Comparison of concentration of OPN & HA in patients with OA knee (cases) and controls

	Group	N	Mean	Std. Deviation	P value
OPN	Control	30	611.050	207.5941	**0.014***
	Cases	60	984.912	804.0174	
HA	Control	30	1.87573	0.480887	**<0.001****
	Cases	60	2.58292	0.908696	

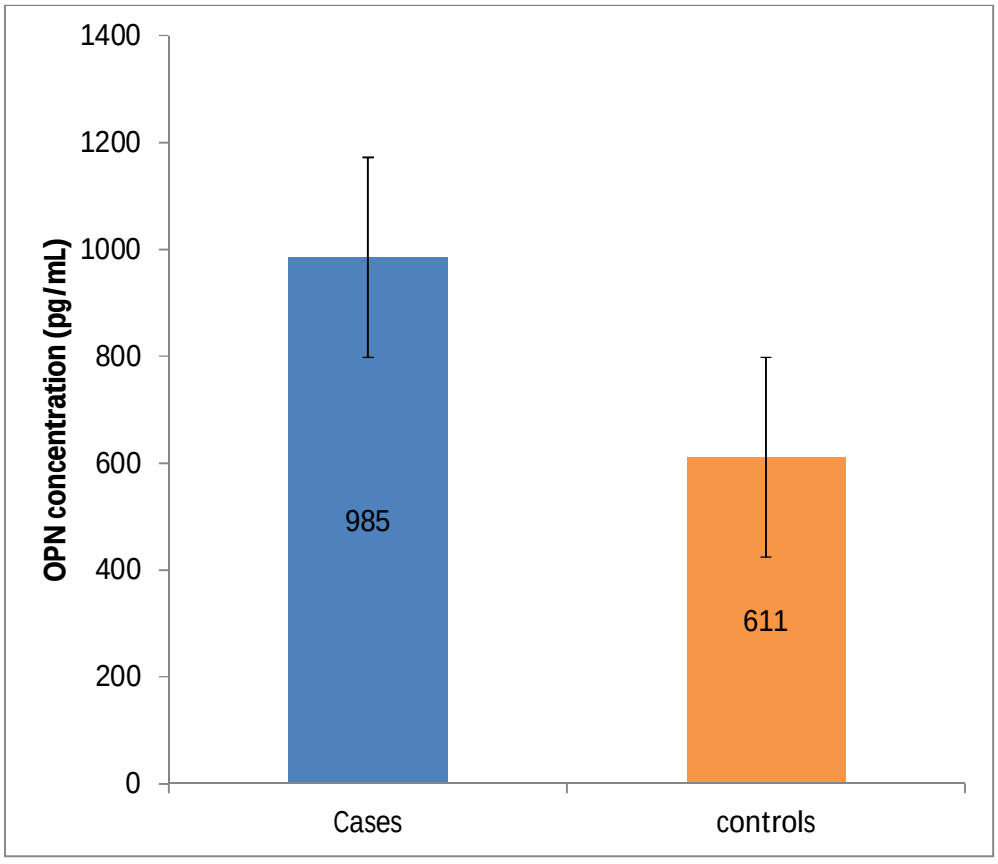
** HIGHLY SIGNIFICANT
*SIGNIFICANT

In Table-6., the mean and standard deviation of the OPN values in patients with OA knee and controls are tabulated. The mean OPN concentration in patients with OA knee was 984.91+/−804pg/mL while in controls it was 611.05+/−207.59 pg/mL. The difference in OPN values between OA knee patients and controls was statistically significant (p=0.014) as shown in Graph1.

Table-6 shows the mean and standard deviation for the values of HA among the cases and controls. The mean HA in OA knee patients was 2.58+/−0.90ng/mL while in controls it was 1.87+/−0.48 ng/mL. There was a highly significant difference in the value of HA in cases and controls with a p-value of <0.001** as shown in Graph-2.

GRAPH-1

Comparison of concentration of OPN in patients with OA knee (cases) and controls



GRAPH-2

Comparison of concentration of HA in patients with OA knee (cases) and controls

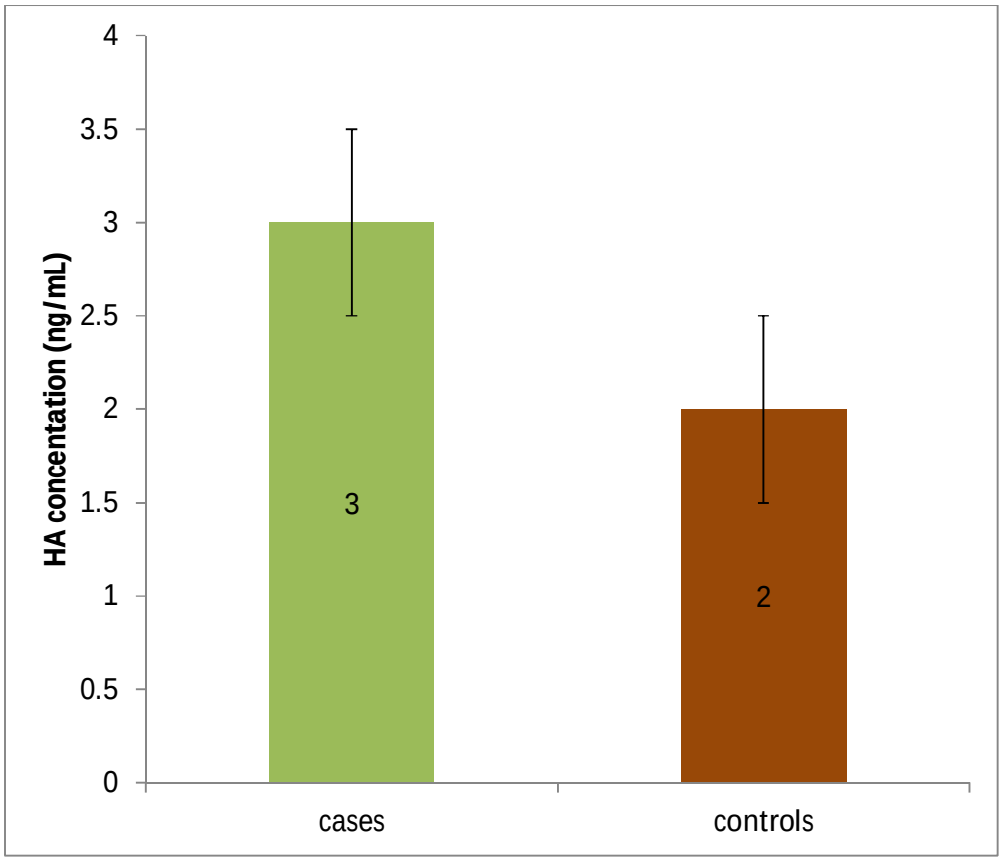


TABLE-7

Comparison of OPN levels with various K/L grades of OA among cases

K/L-GRADE	N	Osteopontin(pg/mL)		p- value	95%Confidence Interval for Mean	
		Mean	Std. Deviation		Lower Bound	Upper Bound
2	9	558.489	107.1066	**0.022***	476.159	640.818
3	37	915.238	693.5673		683.991	1146.485
4	14	1443.179	1108.5290		803.133	2083.224

*SIGNIFICANT

K/L Grade- 2,3,4 & OPN level

Table-7 shows the comparison of plasma osteopontin levels in relation to radiological K/L grading of OA in the present study and it was found that the mean plasma OPN levels in K/L grades 2, 3 & 4 were 558.48+/−107.10pg/mL, 915.238+/−693.56pg/mL and 1443.179+/−1108.52pg/mL This implies that the plasma OPN levels were significantly higher in K/L grades 2, 3 & 4(p=0.022) as shown in Graph-3.

GRAPH-3

Comparison of OPN levels with various K/L grades of OA among cases

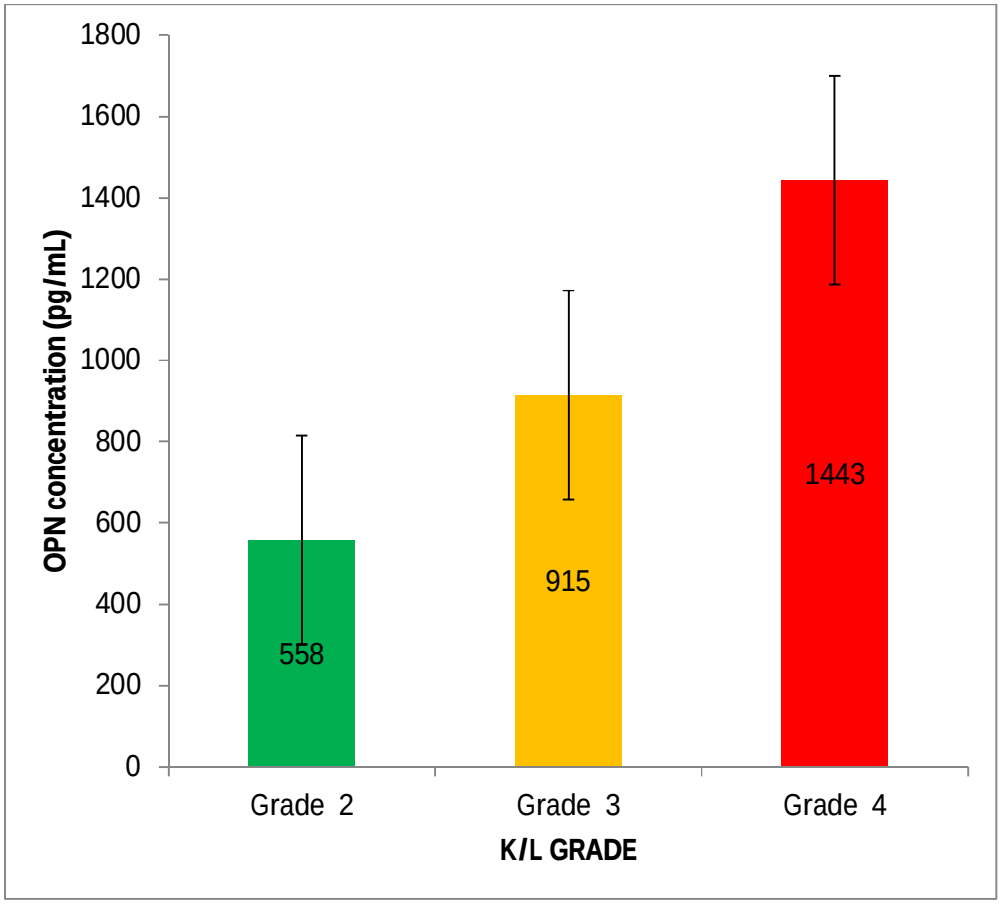


TABLE-8

Comparison of HA levels with various K/L grades of OA among cases

K/L-GRADE	N	Hyaluronic acid(ng/mL)Mean	Std. Deviation	p-value	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
2	9	1.74178	0.530247	**0.006****	1.33419	2.14936
3	37	2.67124	0.998217		2.33842	3.00407
4	14	2.89021	0.448811		2.63108	3.14935

K/L Grade- 2,3,4 & HA level, ** Highly significant

Table-8 shows the comparison of serum HA levels in relation to radiological K/L grading of OA in the present study and it was found that the mean serum HA levels in K/L grades 2, 3 & 4 were1.74+/−0.53 ng/mL, 2.67+/−0.99ng/mL and 2.89+/− 0.44ng/mL .This implies that the serum HA levels were significantly higher in K/L grades 2, 3 & 4 (p=0.006) as shown in Graph-4.

GRAPH-4

Comparison of HA levels with various K/L grades of OA among cases

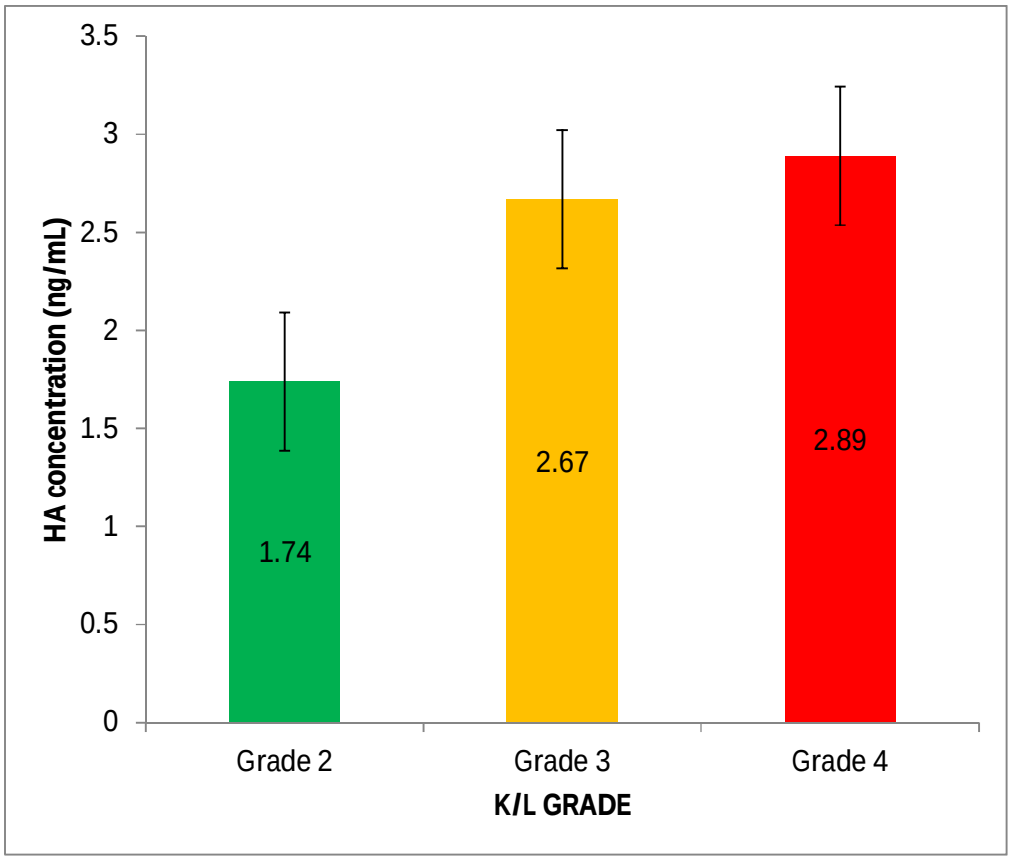


TABLE-9

Pearson correlation coefficient between OPN, HA and K/L grade in the study population

		K/L grade
OPN	**Pearson Correlation**	0.349**(**)**
	Sig. (2-tailed)	**0.006****
	N	60
HA	**Pearson Correlation**	0.358**(**)**
	Sig. (2-tailed)	**0.005****
	N	60

** Correlation is significant at the 0.01 level (2-tailed).

Table-9 shows the Pearson correlation coefficient on variables OPN and K/L grade. In order to measure the strength of linear relationship between OPN and K/L grade, Pearson correlation was done and it was observed that there was a positive linear relationship between OPN and K/L grade with r=0.349, p-value=0.006

Pearson correlation coefficient was done on variables HA and K/L grade in order to measure the strength of linear relationship between the two variables as tabulated. It was observed that as K/L grade increases the concentration of HA also increases, indicated by a positive linear relationship with r=0.358, p-value=0.005

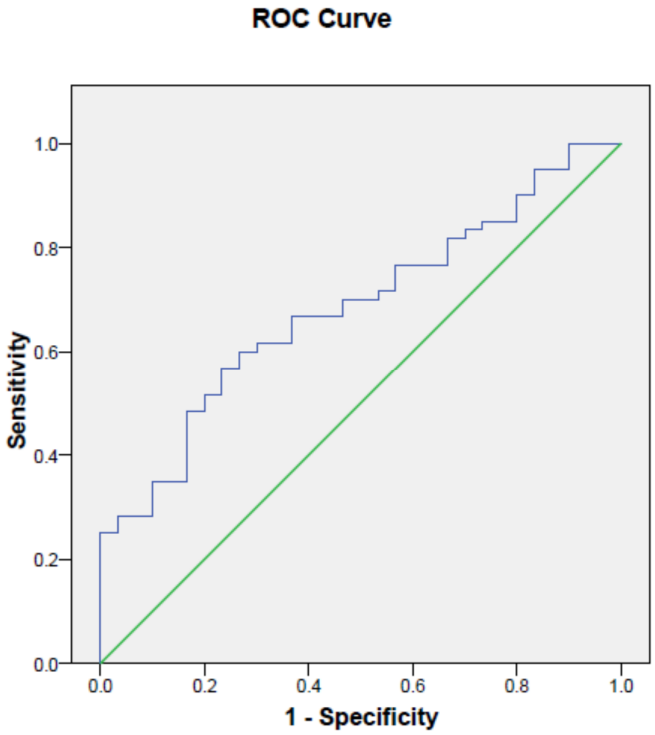
TABLE-10

Pearson correlation coefficient between OPN and HA in the study population

		HA
OPN	**Pearson Correlation**	0.149
	Sig. (2-tailed)	0.256
	N	60

Table-10 shows the Pearson correlation coefficient between the variables OPN and HA. This was done to find out whether there is any linear relationship between these variables. In the present study it was found that there was no statistical significance p-value=0.256.

ROC curve of OPN for analysis of the diagnostic values of plasma OPN in OA patients

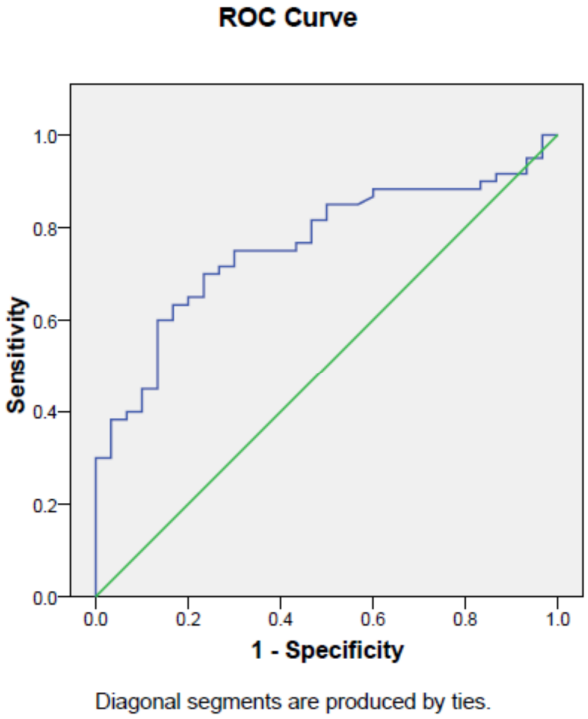


AREA UNDER THE CURVE - OPN

Area	Std. Error(a)	Asymptotic Sig.(b)	Asymptotic 95% Confidence Interval	
0.683	0.056	0.005	0.572	0.793

According to the data of 60 patients with varying grades of OA knee and 30 normal subjects as controls ROC curve was plotted. When 617.95pg/mL was set as cut-off level of plasma OPN, the sensitivity to predict susceptibility of patients for developing OA knee was 67% and the specificity was 63%.The area under the curve for OPN was 0.683. Hence OPN can be used as a biomarker to assess the severity of OA knee.

ROC curve for HA for analysis of the diagnostic values of serum HA in OA patients



AREA UNDER THE CURVE - HA

Area	Std. Error(a)	Asymptotic Sig.(b)	Asymptotic 95% Confidence Interval	
0.761	0.051	0.000	0.662	0.860

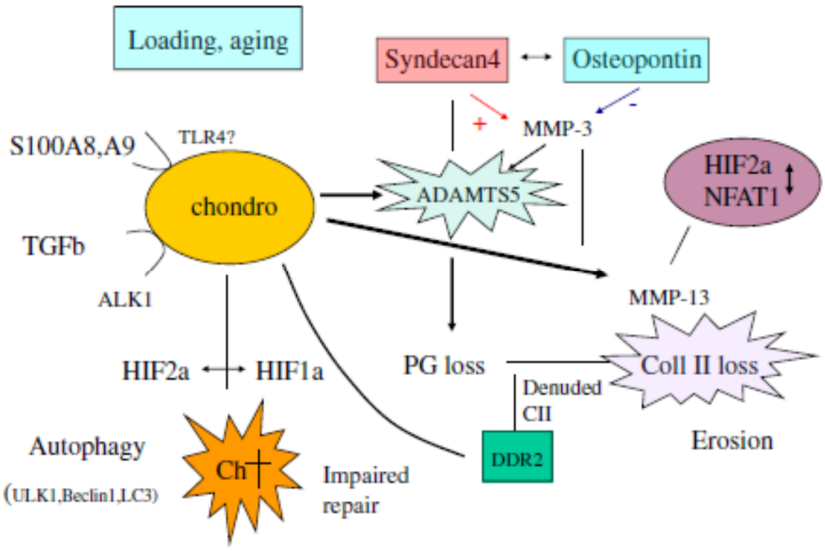
When 2.06ng/mL was set as the cut-off level of serum HA, the sensitivity to predict patients with OA knee was 75% and the specificity was 70%. The area under the curve (AUC) was 0.761 for HA . This implies that HA can be used as a biomarker to assess the cartilage damage in OA knee.

Discussion

DISCUSSION

OA knee is a chronic degenerative disease with multiple pathogenic pathways of chondrocyte triggering and downstream transcription factors, which leads to final common pathway of activation of MMP-13 mediated type-II collagen breakdown which ultimately causes cartilage erosion. OPN also has a crucial role in MMP-13 activation leading to cartilage destruction and release of HA from the cartilage. Finally impairment of regenerative capacity of the cartilage occurs due to cell death caused by deranged autophagy[169] (as shown in the figure below). Till date only plain x-rays are available to assess the severity of OA in the knee joint. Recently newer biomarkers on detecting cartilage matrix synthesis and degradation are gaining clinical importance in the diagnosis of severity of OA knee and also recent advances in the proteomics and microRNA have enabled detection of newer OA biomarkers.

Pathways of OA cartilage pathology



In the present study, the plasma concentration of OPN was evaluated to assess its utility as a biological marker in patients with OA knee and to correlate with radiological grade and to assess, if osteopontin contributes to the pathogenesis of the degenerative process of osteoarthritis by stimulating MMP13 and thereby increasing hyaluronic acid levels in the serum in patients with OA knee.

Insignificant p-values were obtained for variables viz., age, gender, height, systolic and diastolic BP while comparing the cases and controls. This implies that the cases and controls were perfectly matched with respect to the confounding variables. There is a statistically significant difference among cases and controls with respect to weight and BMI. This indicates that obesity has a significant role in pathogenesis of OA knee.

The common secondary causes of OA knee- post traumatic was ruled out by history and the other most common cause the calcium pyrophosphate deposition (CPPD) disease in elderly by estimation of calcium and phosphorus. In this study the levels of serum calcium and phosphorus were normal in both the cases and controls implying that the secondary causes of OA in the study groups has been excluded.

In this study the plasma OPN concentrations were significantly higher in patients with OA knee when compared to controls. Significant differences in OPN concentrations were observed among the cases when they were classified into three groups (K/L grade2, 3 &4) based on their radiological findings. An

increase in concentration of plasma OPN with advancing radiological grade was observed among the patients with OA knee. The OPN levels did not vary significantly with gender in both the study groups in this study. Also a positive correlation was observed between OPN and K/L grade in this study indicating that as K/L grade advances the OPN concentration also increases. Hence plasma OPN level significantly correlates with the severity of the disease. Similar results were obtained by Honsawek et al[126]. Increase in OPN level among cases when compared to controls and increase in osteopontin levels with radiological grade 3 and 4 when compared to K/L grade 2 were also observed by Honsawek et al[126].

HA is produced by cells of ECM and is thought to play a role in cell signalling[170]. Degradation and turnover of ECM results in the release of HA and its fragments into the systemic circulation and endocytosis via a specific HA receptor[141] and they are cleared by the liver and lymphatics. Increased HA concentration in the inflamed synovium has been suggested to contribute to joint stiffness & edema in the arthritic joint[143]. The synovial inflammation and cartilage degradation result in the release of HA into the circulation there by increasing the serum levels. Hence serum HA measurements maybe used to assess the amount of cartilage damage in OA knee.

In the present study the serum HA concentrations were significantly higher in patients with OA knee when compared to controls. Significant differences in HA concentrations were observed among the cases when they

were classified into three groups (K/L grade2,3&4) based on their radiological findings. An increase in concentration of serum HA with advancing radiological grade was observed among the patients with OA knee. This increase in HA level with radiological grade was statistically significant. The HA levels did not vary significantly with gender in both the study groups in this study. Also a positive correlation was observed between HA and K/L grade in this study indicating that as K/L grade advances the HA concentration also increases. Hence HA levels significantly correlates with the severity of the disease and the amount of cartilage damage. Similar result was reported by Sharif et al[171]- increased serum HA levels was observed in patients with late stages of OA than in patients with early stages of OA. A positive correlation was demonstrated between serum HA level and the amount of involved cartilage by another study[172].

Hence in the present study statistically significant increase in concentration of both OPN & HA with respect to radiological grade and a positive correlation between OPN and K/L grade & HA and K/L grade implies that OPN has a significant role in activating MMP-13 causing degradation of articular cartilage and release of HA into the circulation in osteoarthritis. Hence OPN and HA can be used in combination as biomarkers to assess the severity of the disease.

In the present study the correlation between OPN and HA did not show any statistical significance implying that HA level increase is contributed by

many factors other than OPN. OPN and many inflammatory mediators are stimulated in OA knee by various pathways but finally they merge to stimulate MMP-13 which causes destruction of the cartilage thereby releasing HA in systemic circulation.

In the present study analysis of diagnostic value of plasma OPN and serum HA was done by plotting Receiver Operator Characteristic (ROC) curve. Hence both increase in plasma OPN (cut-off value-617.95pg/mL) and increase in serum HA (cut-off value 2.06ng/mL) can be used as biomarkers to assess the severity of the disease and cartilage damage in patients with OA knee.

Conclusion

CONCLUSION

OA knee is a chronic degenerative joint disease leading to joint disability. The disease is emerging as a major health problem and a threat to normal healthy life in recent years. Early diagnosis of OA may help to overcome this problem. For this biomarkers carefully chosen based on our knowledge on pathogenic mechanisms, can be used to predict and assess the severity of OA cartilage. This can help in modulating drug therapy against the specific pathogenic pathway. Till now only x-rays are in use to assess the severity of OA in the knee joint. OPN and HA are the two biomarkers evaluated in the present study to assess the disease progression in OA knee joint.

In the present study there was a significant higher concentrations of OPN and HA in patients with OA knee than in the controls. The concentration of OPN and HA increases as the radiological grade increases while the gender differences cause no significant changes. Hence OPN and HA can be used as biomarkers to assess the disease progression and cartilage damage respectively.

Extensive analysis of different set of markers in large number of patients with OA knee and following them prospectively may help in identifying the markers that are independent predictors of outcome. These biomarkers may serve as a good target for development of drugs to treat OA in the near future.

Limitation of the study

LIMITATIONS OF THE STUDY

1. The sample size was not large enough to arrive at definitive conclusions.
2. The investigation was performed on only those patients who attended Orthopaedics Out-patient department, Rajiv Gandhi Government General Hospital, a tertiary care centre, for evaluation or treatment of knee OA which might not be true representative of patient presenting with OA knee.
3. An important critical issue that limit the use of osteopontin as a specific marker for osteoarthritis is that the osteopontin level is also increased in a variety of cancers.
4. The determination of an accurate cut-off value for circulating osteopontin level is another critical issue in its clinical application. Further studies with standardized assay system in a large population are required to gain insight into the potential utility of osteopontin.

Scope for further studies

FUTURE PROSPECT OF THE STUDY

In OA knee all the major pathogenic mechanisms that cause destruction of cartilage finally merge into one common pathway i,e. stimulation of the Matrix Metalloproteinase-13(MMP-13) which ultimately causes destruction of the cartilage. Hence study can be planned to estimate MMP-13 levels among OA patients and controls to assess the prognostic efficacy of the same in the future to assess the disease progression.

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Annexures

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI 600 003

EC Reg.No.ECR/270/Inst./TN/2013
Telephone No.044 25305301
Fax: 011 25363970

CERTIFICATE OF APPROVAL

To

Dr.P.Nirmaladevi
II Year PG in MD (Bio-Chemistry)
Institute of Bio-Chemistry
Madras Medical College
Chennai 600 003

Dear Dr.P.Nirmaladevi,

The Institutional Ethics Committee has considered your request and approved your study titled **"CORRELATION OF PLASMA OSTEOPONTIN WITH RADIOLOGICAL GRADING IN PATIENTS WITH OSTEOARTHRITIS IN THE KNEE JOINT "** NO.11012015.

The following members of Ethics Committee were present in the meeting hold on 20.01.2015 conducted at Madras Medical College, Chennai 3.

1. Dr.C.Rajendran, MD	:Chairperson
2. Dr.R.Vimala,MD.,Dean,MMC,Ch-3	: Deputy Chairperson
3. Prof.B.Kalaiselvi,MD.,Vice Principal,MMC,Ch-3	: Member Secretary
4. Prof.R.Nandhini,MD.,Inst.of Pharmacology,MMC	: Member
5. Prof.P.Ragumani, MS., Professor, Inst.of Surgery,MMC	: Member
6. Prof.K.Ramadevi, Director , Inst.of Bio-Chem.MMC	: Member
7. Prof.Saraswathy,MD.,Director,Pathology, MMC	: Member
8. Prof.Md.Ali, MD., DM.,Prof.&HOD of Medl.GE,MD.MMC	: Member
9. Thiru S.Rameshkumar	: Lay Person
10.Thiru S.Govindasamy, BA., BL.,	: Lawyer
11.Tmt.Arnold Saulina, MA., MSW.,	: Social Scientist

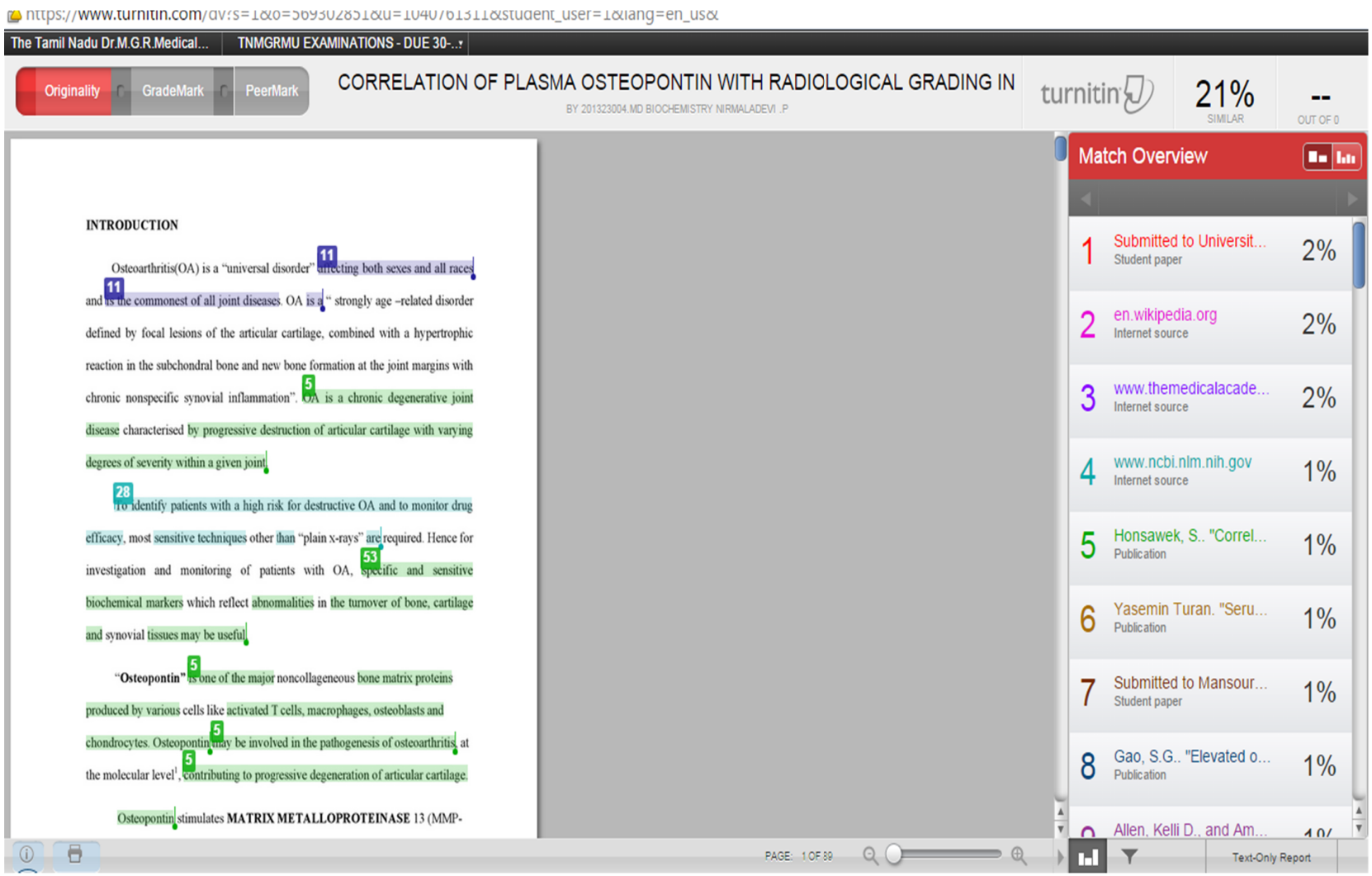
We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

Member Secretary - Ethics Committee

Sys 2

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE
CHENNAI-600 003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INTRODUCTION
Osteoarthritis(OA) is a "universal disorder" affecting both sexes and all races and is the commonest of all joint diseases. OA is a " strongly age –related disorder defined by focal lesions of the articular cartilage, combined with a hypertrophic reaction in the subchondral bone and new bone formation at the joint margins with chronic nonspecific synovial inflammation". OA is a chronic degenerative joint disease characterised by progressive destruction of articular cartilage with varying degrees of severity within a given joint.
To identify patients with a high risk for destructive OA and to monitor drug efficacy, most sensitive techniques other than "plain x-rays" are required. Hence for investigation and monitoring of patients with OA, specific and sensitive biochemical markers which reflect abnormalities in the turnover of bone, cartilage and synovial tissues may be useful.
"Osteopontin" is one of the major noncollageneous bone matrix proteins produced by various cells like activated T cells, macrophages, osteoblasts and chondrocytes. Osteopontin may be involved in the pathogenesis of osteoarthritis at the molecular level[1], contributing to progressive degeneration of articular cartilage.
Osteopontin stimulates MATRIX METALLOPROTEINASE 13 (MMP-
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INFORMATION SHEET

Title: Correlation of plasma osteopontin with radiological grading in patients with osteoarthritis in the knee joint.

Investigator : **Dr.P.Nirmaladevi**
Post Graduate,
Institute of Biochemistry,
Madras Medical College,
Chennai- 600 003.

Guide : **Dr. R. Chitraa**,
Professor,
Institute of Biochemistry,
Madras Medical College,
Chennai- 600 003.

Osteoarthritis is a universal and most common joint disease affecting both males and females equally. Hence to identify patients with a high risk for destructive OA and to monitor drug efficacy more sensitive techniques than plain X- ray are needed. The purpose of the present study is to correlate concentration of osteopontin levels in plasma of patients with knee osteoarthritis and to evaluate the possible correlation with the radiographic grading of knee osteoarthritis.

Hence I am doing this study titled **"Correlation of plasma osteopontin with radiological grading in patients with osteoarthritis in the knee joint**" attending Rajiv Gandhi Govt. General Hospital, Chennai. To do this study , I need to collect 6ml of blood from 60 knee osteoarthritis patients and 30 healthy subjects, while collecting blood there will not be any side effects.

Your identity will be confidential throughout the study and during publication or presentation in any clinical journals.

Participation in this study is purely voluntary. You can withdraw from the study at any time. Your decision will not result in any loss of benefits to which you are otherwise entitled. The results of the study will be intimated to you. Kindly sign in this information sheet and consent form, if you have willingness to participate in this study,

Signature of investigator
Place:
Date:

Signature of participant
Thumb Impression

PATIENT CONSENT FORM

TITLE OF THE STUDY **: CORRELATION OF PLASMA OSTEOPONTIN WITH RADIOLOGICAL GRADING IN PATIENTS WITH OSTEOARTHRITIS IN THE KNEE JOINT".**

Name : Date :

Age : OP No

Sex : Project Patient No :

Documentation of the informed consent

I _____ have read the information in this form (or it has been read to me). I was free to ask any questions and they have been answered. I hereby give my consent to be included as a participant in "**CORRELATION OF PLASMA OSTEOPONTIN WITH RADIOLOGICAL GRADING IN PATIENTS WITH OSTEOARTHRITIS IN THE KNEE JOINT".**

1. I have read and understood this consent form and the information provided to me.
2. I have had the consent document explained to me.
3. I have been explained about the nature of the study.
4. I have been explained about my rights and responsibilities by the investigator.
5. I have been informed the investigator of all the treatments I am taking or have taken in the past _____ months including any native (alternative) treatment.
6. I have been advised about the risks associated with my participation in this study.
7. I agree to cooperate with the investigator and I will inform him/her immediately if I suffer unusual symptoms.
8. I have not participated in any research study within the past _____ month(s).
9. I am aware of the fact that I can opt out of the study at any time without having to give my reason and this will not affect my future treatment in this hospital.
10. I am also aware that the investigator may terminate my participation in the study at any time, for any reason, without any consent.
11. I hereby give permission to the investigators to release the information obtained from me as result of participation in this study to the sponsors, regulatory authorities, Govt. agencies, and IEC. I understand that they are publicly presented.
12. I have understood that my identity will be kept confidential if my data are publicly presented.
13. I have had my questions answered to my satisfaction.
14. I have decided to be in the research study.

I am aware that if I have any question during this study, I should contact the investigator. By signing this consent form I attest that the information given in this document has been clearly explained to me and understood by me, I will be given a copy of this consent document.

For participants:
Name and signature / thumb impression of the participant (or legal representative if participant in competent/For age 10-17 yrs-Name& signature of the parent/guardian.)

Name _____ Signature_____ Date_____

Name and Signature of impartial witness (required for illiterate patients):

Name _____ Signature_____ Date_____

Address and contact number of the impartial witness:

Name and Signature of the investigator or his representative obtaining consent:

Name _____ Signature_____ Date_____

ஆராய்ச்சி தகவல் தாள்

தலைப்பு:

பிளாஸ்மா ஆஸ்டியோபான்டினை முழங்கால் கீல்வாதம் உள்ள நோயாளிகளின் கதிர்வரைவுகளை தரவாரியாகப் பிரித்து தொடர்பு படுத்தி கண்டறியும் ஓர் ஆய்வு.

ஆராய்ச்சியாளர் : **மரு. பெ. நிர்மலாதேவி,**
பட்ட மேற்படிப்பு மருத்துவ மாணவி,
உயிர்வேதியியல் உயர்நிலைத்துறை,
சென்னை மருத்துவக் கல்லூரி மருத்துவமனை,
சென்னை - 600003.

ஆராய்ச்சி மேற்பார்வையாளர் : **மரு. ரா. சித்ரா,**
பேராசிரியர்,
உயிர்வேதியியல் உயர்நிலைத்துறை,
சென்னை மருத்துவக் கல்லூரி மருத்துவமனை,
சென்னை - 600003.

கீல்வாதம் என்னும் மூட்டுநோய் உலக அளவில் ஆண் மற்றும் பெண் இருவரையும் சமமாக பாதிக்கக்கூடிய ஒரு நோயாகும். எனவே இந்த நோயால் ஏற்படக்கூடிய அதிக ஆபத்து மற்றும் அழிவை கண்டறியவும், மேலும் இந்நோய்க்கு அளிக்கப்படும் மருந்தின் செயலாற்றத்தை அறிந்து கொள்ளவும் ஊடுகதிர் தவிர பிற முக்கிய நுட்பங்களை கண்டறிய வேண்டியது அவசியமாகிறது. எனவே பளாஸ்மா ஆஸ்டியோபான்டினை முழங்கால் கீல்வாதம் உள்ள நோயாளிகளின் கதிர்வரைவுகளை தர வாரியாகப் பிரித்து தொடர்புபடுத்தி கண்டறிவதே இந்த ஆய்வின் நோக்கமாகும்.

எனவே சென்னை இராஜீவ்காந்தி அரசு பொது மருத்துவமனைக்கு வரும் முழங்கால் கீல்வாதம் உள்ள நோயாளிகளின் கதிர்வரைகளை தரவாரியாகப் பிரித்து அதனுடன் பிளாஸ்மா ஆஸ்டியோபான்டின் செறிவை தொடர்புபடுத்தி கண்டறியும் ஆராய்ச்சியில் ஈடுபட்டுள்ளேன்.

இதற்கு முழங்கால் கீல்வாதம் உள்ள 60 நோயாளிகளிடமும், அவர்களின் வயதிற்கு ஏற்றார் போலுள்ள 30 நோயற்ற ஆரோக்கியமான நபர்களிடமும் 5 மி.லி. இரத்தம் எடுத்து ஆராய்ச்சிக்கு உட்படுத்த உள்ளேன்.

தங்களிடமிருந்து ஊசியின் மூலம் 5 மி.லி. இரத்தம் எடுப்பதனால் எந்தவிதமான பக்க விளைவுகளும் ஏற்படாது என உறுதி அளிக்கின்றேன்.

தாங்கள் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்புகிறோம். இதில் பங்கு பெறுவதினால் நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்கு உள்ளாகாது என்பதையும் கூடுதல் செலவீனம் ஏற்படாது என்பதையும் தெரிவித்துக் கொள்கிறோம்.

முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயர் மற்றும் அடையாளங்கள் வெளியிடப்படாது என்பதை தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களின் விருப்பத்தின் பேரில் தான் இருக்கிறது. எந்நேரமும் இதிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியின் முடிவுகளை தங்களுக்கு அறிவிப்போம் என்பதை தெரிவித்துக் கொள்கிறோம்.

நீங்கள் இந்த ஆராய்ச்சியில் பங்குபெற விருப்பம் இருப்பின் இந்த தகவல்தாள் மற்றும் ஆராய்ச்சி ஒப்புதல் படிவத்திலும் கையொப்பம் இடுமாறு கேட்டுக் கொள்கிறோம்.

ஆராய்ச்சியாளர் கையொப்பம்

பங்கேற்பாளர் கையொப்பம்
/இடது கைவிரல் ரேகை

இடம் :

தேதி :

நோயாளியின் ஒப்புதல் படிவம்

தலைப்பு:

பிளாஸ்மா ஆஸ்டியோபான்டினை முழங்கால் கீல்வாதம் உள்ள நோயாளிகளின் கதிர்வரைவுகளை தரவாரியாகப் பிரித்து தொடர்பு படுத்தி கண்டறியும் ஓர் ஆய்வு.

பங்கேற்பாளர் பெயர் : புற / உள் நோயாளி எண்:

வயது : பால் : கைபேசி/தொலைபேசி எண் :

முகவரி : ஆராய்ச்சி சேர்க்கை எண் :

.....ஆகிய நான் மரு.பெ.நிர்மலாதேவி, பட்டமேற்படிப்பு, மருத்துவ மாணவி, உயிர்வேதியியல் உயர்நிலைத் துறை, சென்னை மருத்துவக் கல்லூரி, சென்னை. மேற்கொள்ளும் ஆராய்ச்சியில் பங்கேற்க எந்தவித நிர்பந்தமின்றி, முழு சுதந்திரத்துடன் சுய நினைவுடன் முழு மனதுடன் சம்மதம் தெரிவிக்கின்றேன்.

இந்த ஆராய்ச்சி பற்றிய தகவல் தாளை பெற்றுக் கொண்டு அதன் முழு விவரங்களையும், நோக்கங்களையும் மருத்துவரின் மூலம் புரிந்துக்கொண்டு எனது மருத்துவ குறிப்புகள், பரிசோதனை முடிவுகள் மற்றும் இரத்தத்தை பயன்படுத்திக் கொள்ளவும் மேலும் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின்வாங்கலாம் எனவும் அச்செயலினால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் புரிந்துக் கொண்டு முழு மனதுடன் சம்மதம் தெரிவிக்கின்றேன்.

எனது பரிசோதனை முடிவுகளை அறிவியல் சார்ந்த அமைப்புகள் மற்றும் மருத்துவ இதழ்களில் வெளியிடுவதற்கு முழு மனதுடன் சம்மதிக்கின்றனே.

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்
/இடது கைவிரல் ரேகை

இடம் :

தேதி :

PROFORMA

Date: Sample no: Case/Control:

Name ·

Age/Sex ·

OP/IP No ·

Address ·

Height ·

Weight ·

Body Mass Index ·

Diagnosis ·

Chief presenting complaints ·

Pain in the knee joint ·

Duration ·

Swelling over knee ·

Tenderness ·

Crepitus ·

Effusion ·

Muscle wasting ·

Limited mobility ·

Any other serious medical illness ·

Past History ·

Diabetes ·

Hypertension ·

Personal History ·

Smoking Duration ·

Alcoholism Duration ·

Family History ·

On Examination ·

Conscious ,Oriented

Pallor

Icterus

Pedal oedema

Knee swelling

Mobility

Tenderness

Vitals: PR: BP:

CVS:

RS:

Abdomen:

DIAGNOSIS:

INVESTIGATIONS:

Serum calcium

Serum phosphorus

Plasma osteopontin

Serum hyaluronic acid

X-ray knee joint - antero-posterior view